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PREPARATION OF GALACTOSE.

By E. P. CLARK.

(From the Polarimetry Section, United States Bureau of Standards, Washington.)

CORRECTION.

The accompanying equation is to replace the equation on page 415, Vol. XLVI, No. 2, April, 1921, line 12. The slip is arranged to be pasted over the place occupied by the equation at present.

Recently, an unpublished method, originated in the Bureau of Chemistry, has been used quite successfully by many workers. However, it is not generally known. The method which we have worked out in order to meet the demands made upon this Bureau has the advantages over the above mentioned methods of giving more uniform results, higher yield, and lower cost, and is reported here that it may be useful to those requiring a supply of this sugar.

1,500 gm. of lactose are dissolved in 3,750 cc. of hot water containing 75 gm. of concentrated sulfuric acid. The solution is brought to a boil and then simmered for 2 hours. A thin paste of barium carbonate is then added to the hot solution until it reacts neutral to Congo paper. The precipitate of barium sulfate is allowed to settle over night, after which as much as possible of the supernatant liquid is drawn off. This liquid is filtered through a thin layer of active carbon placed on moistened filter paper in a Buchner funnel. When all has passed through, the precipitate is placed on the filter, drained as dry as possible, and finally washed by drawing a little water through it. The filter, prepared with a small amount of carbon, as outlined, clarifies the solution and at the same time prevents the sulfate from passing through and gives a relatively rapid filtration.

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[1] von Lippmann, E. O., Die Chemie der Zuckerarten, 1904, iii, 698-700.

The filtrate is concentrated under diminished pressure until it has a weight of 1,650 gm. If a refractometer is available, the concentrated syrup should have a refractive index between 1.5120 and 1.5125. The very thick syrup is warmed to between 60 and 70° C. and 250 cc. of ethyl alcohol are dissolved in it by vigorous shaking. The solution is then poured into a beaker or jar and the remaining syrup is washed from the flask with 500 cc. of methyl alcohol. This is done best by adding the methyl alcohol to the flask portionwise, warming, and shaking in a water bath. The whole solution is thoroughly mixed, seeded with some pure galactose crystals, and allowed to crystallize.

The crystallization is generally complete in about 4 days, after which the galactose is filtered off, washed with a little methyl alcohol, then with 85 per cent ethyl alcohol, and finally with 95 per cent alcohol. It is then dried. The yield of this crude sugar is about 27 per cent of the lactose taken.

In order to purify the crude galactose, it is dissolved in water, making about a 25 per cent solution. To this is added a few cc. of glacial acetic acid. It is then concentrated under diminished pressure to about 75 per cent total solids, warmed to 60 or 70° C., transferred to a beaker, and 95 per cent alcohol added to saturation. Almost immediately the contents of the flask become solid. After allowing to stand over night, it is filtered from the mother liquor, washed, and dried. The yield is generally 82 to 83 per cent of the crude sugar taken.

A thoroughly dried sample of this recrystallized product had the following properties:

Ash = 0.034 per cent.
$[\alpha]_{D}^{23}$ = 80.4° in 10.617 per cent aqueous solution.
$[\alpha]_{\lambda 5461\,\text{Å.u.}}^{20}$ = 95.8° " 10.617 " " " "

The values are only given, however, to identify the product. Work upon the thorough purification and the accurate determination of its specific rotation is in progress.

A NOTE ON THE ABSORPTION OF CALCIUM SALTS IN MAN.

By EDWARD H. MASON.

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(Received for publication, April 7, 1921.)

Until recently, calcium salts, especially the lactate, have been extensively employed as hemostatics. Their use has been largely empirical for there is little experimental evidence to show that the calcium content of the blood can be influenced by their oral administration when its calcium concentration is already normal.

TABLE I.

Case.	Dose	Plasma calcium (as Ca) per 100 cc.				Maximum increase.
		Before.	1 hr	2 hrs.	3 hrs	
		mg	*mg.*	*mg.*	*mg.*	
1	Calcium lactate 5 gm.	10.2	12.8	11.2	13.0	+2.6
2	" " 5 "	13.0	13.0	13.0	13.0	0
3	" " 5 "	12.0	11.7	12.0	12.2	0
1	Calcium lactate 5 gm. + 25 cc. cod liver oil.........	10.4	10.0	10.0	9.8	0
2	" " "	13.4	14.8	14.0	14.0	+1.4
3	" " "	13.0	14.6	12.8	13.1	+1.6
4	Calcium chloride 5 gm.. .. .	14.8	16.2	16.0	15.2	+1.4
5	" " 5 "	11.2	13.2	13.8	Lost.	+2.6
6	" " 5 "	11.0	15.4	14.8	14.8	+4.4
7	" " 5 "	12.6	15.0	15.2	15.2	+2.6
8	Calcium lactate 5 gm. + 25 cc. 0.05 N HCl..................	11.4	11.2	9.8	9.4	0
9	" " "	8.4	10.4	9.6	9.6	+2.0
10	Calcium chloride 5 gm. + 25 cc. 0.05 N HCl...........	11.2	13.2	13.2	13.6	+2.4
11	" " "	11.6	12.4	12.8	12.8	+1.2

Halverson, Mohler, and Bergeim (1) showed that in certain cases of uremia with a low blood calcium the latter could be appreciably affected by the oral administration of calcium lactate. Denis and Minot (2) also agreed that it is usually impossible to influence the calcium content of the plasma by calcium lactate ingestion.

A short series of observations has been completed on normal subjects. The determinations were made by Lyman's (3) technique, using citrated plasma as advocated by Halverson, Mohler, and Bergeim. Two salts, the lactate and the chloride, were used. Both were administered in 5 gm. doses in 300 cc. of water on a fasting stomach. The plasma values before, and each hour afterwards for 3 hours, were determined. Likewise, both salts were dissolved in 25 cc. of 0.05 N HCl, and given with 250 cc. of water. Also the lactate was given with 25 cc. of cod liver oil.

The results are shown in Table I.

From the results in Table I it would appear that a single large dose of calcium lactate given in either of the above three forms little influences the plasma values. Calcium chloride seems to be absorbed better and shows more consistent increases in the plasma values. Its solution in weak hydrochloric acid does not affect its rate of absorption.

BIBLIOGRAPHY.

1. Halverson, J. O., Mohler, H. K., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 171.
2. Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1920, xli, 357.
3. Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.

DINITROSALICYLIC ACID: A REAGENT FOR THE ESTIMATION OF SUGAR IN NORMAL AND DIABETIC URINE.

BY JAMES B. SUMNER.

WITH THE ASSISTANCE OF V. A. GRAHAM.

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(Received for publication, March 26, 1921.)

The methods used for the quantitative determination of sugar in urine, including the polariscope, give good results when the amount of sugar present is considerable, but there is always a point where the urine contains so little sugar that the results are dubious. Special methods have been published for the determination of sugar in normal urine. Many of these give results that are too high, and others, like the method of Benedict and Osterberg,[1] are too long for clinical work.

It appeared that some reagent might be found which could be used both qualitatively and quantitatively and which could be applied to both normal and diabetic urine. The author believed that the most suitable substance would be a compound similar to picric acid in that it would be reduced by glucose to form a highly colored nitroamino compound, but dissimilar to picric acid in that it would neither form colored compounds with acetone or creatinine nor be reduced by urinary constituents other than the sugars.

Two nitro compounds have been found which meet most of these requirements; these are 4, 6-dinitroguaiacol and 3, 5-dinitrosalicylic acid.[2] Both of these compounds can be used successfully for the estimation of glucose in diabetic urine, but while they are not reduced in the presence of sodium carbonate by any urinary constituent yet tried, with the exception of the reducing sugars, they are reduced by such substances as uric acid and polyphenols when glucose is also present. Moreover, they are reduced by the urine as a whole even after the sugar present has been destroyed. The results obtained by the use of these reagents for the sugar in normal urine may be as much as 100 per cent too high, or even more in some cases.

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[1] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

[2] Hübner, H., *Ann. Chem.*, 1879, cxcv, 45.

The author with Miss V. A. Graham recently read a paper on the use of dinitroguaiacol at the meeting of the American Society of Biological Chemists and will not describe the use of dinitroguaiacol in this paper because he believes dinitrosalicylic acid is a far better reagent. While both compounds, under the same conditions of heating and alkalinity, give approximately the same results with normal urine, dinitrosalicylic acid possesses the advantages of being more soluble in the form of its sodium salt, of being lighter in color, and cheaper to prepare. In addition dinitrosalicylic acid is an excellent protein precipitant and on this account can probably be used for the determination of blood sugar.

Using dinitrosalicylic acid as a reagent, the author has found a way to obtain the true values for the sugar in normal urine as follows: 1 cc. of urine is heated in boiling water with 1 cc. of 3 per cent sodium hydroxide for 15 minutes. This treatment destroys the reducing sugars completely, provided the amount present is less than 1 mg. Either 0.5 or 1 mg. of glucose, depending upon the quantity of glucose estimated to have been present originally, is now added to the cooled solution, after which 1 cc. of dinitrosalicylate solution is added and the test-tube heated for 5 minutes. Any reduction exceeding that given by the added glucose is caused by substances which are not sugars. The reducing value of these substances is subtracted from the total reducing value of the urine, giving the value for sugar by difference. It has been found that the quantity of glucose added must be approximately equal to the amount destroyed by heating with alkali, otherwise an error is introduced.

That glucose when present in small amounts can be completely destroyed by heating with alkali has been carefully proved, using the sensitive copper and phosphomolybdic reagents used by Folin and Wu[3] for the determination of blood sugar. With these reagents it was found that heating 1 cc. of a solution of 1 mg. of glucose with 1 cc. of 3 per cent sodium hydroxide solution for 15 minutes destroyed all but 2 to 3 per cent of the sugar. Heating for 20 minutes destroyed all of the sugar. In practice the author heats the urine with alkali for 15 minutes and uses diluted urine when the reduction due to both sugar and other reducing substances is equivalent to more than 1.5 mg. The reduction due to sugar alone is usually about 60 per cent of the total reduction.

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[3] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

That the material in normal urine destroyed by heating with alkali is mostly sugar has been proved by fermenting urine with yeast. The yeast treatment is as follows: 50 cc. of urine together with a little paraffin and a few glass beads are weighed in a 100 cc. Erlenmeyer flask, acidified with a drop or two of glacial acetic acid if not already acid, and boiled for 5 minutes to remove dissolved toluene. The flask is cooled and water is added to restore the original weight after which the contents are well mixed with half a cake of Fleischmann's compressed yeast. The flask is stoppered and kept in an incubator at 32-33°C. for about 20 hours. Blanks containing pure glucose were fermented occasionally. It was found that with a glucose concentration of 1 mg. per cc. all the glucose was removed.

Table I shows the reduction given by unfermented urine before and after heating with alkali and by fermented urine before and after heating with alkali. A correction has been made for the water content of one-half a cake of yeast. This was found to amount to about 4.5 gm.

These results show plainly that normal urine contains something that reduces dinitrosalicylic acid, that is readily destroyed by heating with sodium hydroxide, and that is largely fermented by yeast. The results show also that the reducing material in normal urine which is not destroyed by heating with alkali is not fermented by yeast.

Shaffer and Hartmann[4] have recently published a paper in which they claim that normal urine contains little or no fermentable sugar. We are obliged to disagree with this statement.

Preparation of 3, 5-Dinitrosalicylic Acid (*the Author's Method*).—Mix 75 gm. of concentrated sulfuric acid with 15 gm. of concentrated nitric acid and cool in ice water. When well cooled add with shaking 15 gm. of salicylic acid in small portions at a time, keeping the preparation well cooled. When all the salicylic acid has been added the material is poured into about 800 cc. of water, cooled for some time, and filtered off by suction. The crystals of dinitrosalicylic acid are dissolved by heating in dilute sodium carbonate solution, filtered, and salted out by adding a large excess of saturated sodium carbonate solution, followed by cooling. The sodium salt is filtered off by suction and washed with saturated sodium carbonate solution. The material is now dissolved in hot water, filtered, and precipitated as the free acid by adding a large excess of strong hydrochloric acid. After cooling, the dinitrosalicylic acid is filtered off, pressed, recrystallized once from a small amount of boiling water, and dried to constant weight at 100°C.

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[4] **Shaffer, P. A., and Hartmann, A. F.**, *J. Biol. Chem.*, **1920-21, xlv, 365.**

TABLE I.

Percentage Reduction of Normal Urine.

Urine No.	Before fermentation.		After fermentation	
	Total reduction.	After heating with alkali.	Total reduction.	After heating with alkali.
1	0.041	0.022	0.022	0.025
2	0.106	0.061	0.065	0.061
3	0.074	0.032	0.034	0.032
4	0.088	0.031	0.044	0.029
5	0.126	0.050	0.061	0.050
6	0.165	0.065	0.087	0.057
7	0.120	0.046	0.053	0.055
8	0.090	0.040	0.053	0.042
9	0.232	0.083	0.123	0.080
10	0.087	0.033	0.034	0.033
Average ..	0.113	0.046	0.058	0.046

Total sugar	0.067	(0.113-0.046)
Fermentable sugar	0.055	(0.113-0.058)
Unfermentable sugar	0.012	(0.058-0.046)

Preparation of Dinitrosalicylate Solution.—Dissolve 2 gm. of dinitrosalicylic acid in about 70 cc. of hot water with the aid of 10 cc. of 20 per cent sodium carbonate solution. The solution is cooled and made up to 100 cc. volume.

Sodium Hydroxide Solutions.—Two solutions of sodium hydroxide are required, containing 1.5 and 3.0 per cent of sodium hydroxide, respectively.

Glucose Solutions.—Solutions containing 1 and 0.5 mg. of glucose per cc. are prepared and preserved with toluene.

Method.

Pipette 1 cc. of urine into a 1.5 by 15 cm. test-tube; add 1 cc. of the 2 per cent dinitrosalicylate solution and 2 cc. of the 1.5 per cent sodium hydroxide solution. Mix, plug with cotton, and heat in boiling water for 5 minutes. Cool and dilute to 25, 50, or 100 cc. volume according to the depth of color. Mix and compare in colorimeter against standard. If the sugar content of the urine is greater than 0.4 per cent the test must be repeated with diluted urine. If the unknown solution contains an appreciable quantity of precipitate it should be centrifuged.

The above procedure gives with normal urine results that are too high because of the reducing action of uric acid and polyphenols. The reduction due to these substances is determined as follows: Pipette into a test-tube 1 cc. of urine and 1 cc. of 3 per cent sodium hydroxide solution. Mix, plug with cotton, and heat in boiling water for 15 minutes. If the total reduction has amounted to more than 1.5 mg. per cc. calculated as glucose, diluted urine must be used. After heating for 15 minutes cool and add 1 cc. of a solution containing either 1 or 0.5 mg. of glucose, according to whether the total reduction of the urine was more or less than 1 mg. per cc. calculated as glucose. Add 1 cc. of 2 per cent dinitrosalicylate solution, mix, plug with cotton, and heat for 5 minutes in boiling water, proceeding as above. After subtracting from the result obtained the amount of glucose added the remainder will be the value for the uric acid and polyphenols. This last is subtracted from the original reducing value of the urine and gives as the difference the value of the sugars. With diabetic urine this procedure is not necessary unless the sugar content is low.

Standard.—Heat 1 cc. of a solution containing 1 mg. of glucose with 1 cc. of the dinitrosalicylate solution and 2 cc. of the 1.5 per cent sodium hydroxide solution for 5 minutes; cool, dilute to 25 cc. volume, and mix. This standard will keep for several hours.

NOTE ON THE GASOMETRIC DETERMINATION OF NITROGEN.

By RAYMOND L. STEHLE.

(From the Laboratory of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)

(Received for publication, April 28, 1921.)

In a recent communication on this subject[1] it was observed that oxygen was liberated to some extent along with nitrogen when the Kjeldahl digest was treated with sodium hypobromite. The procedure there described called for its absorption with alkaline pyrogallate before reading the gas volume. An investigation as to the cause of the liberation of oxygen has shown that the copper sulfate added to hasten the digestion is responsible. Hence, if this substance is omitted, nitrogen only is liberated and there is no need for the pyrogallate treatment. Besides simplifying the manipulation, the gas volume can then be read with more accuracy, due to the fact that one is dealing with a water-clear solution instead of a highly colored one.

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[1] **Stehle, R. L., *J. Biol. Chem.*, 1920–21, xlv, 223.**

THE GASOMETRIC DETERMINATION OF UREA IN URINE.

BY RAYMOND L. STEHLE.

(From the Laboratory of Physiological Chemistry, School of Medicine, University of Pennsylvania.)

(Received for publication, April 29, 1921.)

Several months ago[1] in a communication describing a gasometric method for the determination of total nitrogen, mention was made of the fact that when carried out *in vacuo* the reaction between sodium hypobromite and urea results in the liberation of the theoretical volume of nitrogen. The necessary vacuum is readily obtainable with the Van Slyke apparatus for determining the carbon dioxide content of blood plasma. Some effort was spent at the time in attempting to utilize the reaction for a blood urea method but it became evident that the error introduced by other nitrogenous constituents of blood was greater than permissible.

Recently Youngburg[2] has shown that the urease method for urinary urea may be somewhat simplified by first removing the ammonium salts and then carrying out the urease decomposition and aeration in the manner described by Van Slyke and Cullen.[3] The ammonium salts are removed by shaking the urine with permutit. The idea immediately suggested itself that here was a place to apply the hypobromite reaction. In the older hypobromite methods the results were unsatisfactory because the reaction (using pure urea solutions) was found to give less than the theoretical amount of nitrogen even when allowed to continue for hours, and in addition ammonium salts and other urinary constituents also yielded nitrogen when subjected to the action of hypobromite. With the aid of permutit the ammonium salts may now be eliminated as sources of error. It remained, therefore, to determine the error which might be introduced by other substances present in urine.

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[1] Stehle, R. L., *J. Biol. Chem.*, 1920-21, xlv, 223.

[2] Youngburg, G. E., *J. Biol. Chem.*, 1920-21, xlv, 391.

[3] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.

Assuming for the moment that an average 24 hour specimen of urine contains 15 gm. of urea nitrogen, 0.300 gm. of creatinine nitrogen, and 0.250 gm. uric acid nitrogen, it is evident that if all the nitrogen of the two latter constituents was liberated quantitatively the error introduced would be about 3.7 per cent. In order to determine the actual quantities liberated these two compounds were subjected to the action of hypobromite. It was found that creatinine yielded about one-seventh of its nitrogen while uric acid yielded about one-half. In the case of the latter, however, the evolution of nitrogen takes place slowly and in consequence, by limiting the reaction time to that required for the urea reaction to go to completion, the error need not exceed about 0.3 per cent.

TABLE I.

Urea Nitrogen, per 1 Cc. of Urine.

Hypobromite		Van Slyke and Cullen.		Youngburg.	
mg		*mg.*		*mg.*	
7.60	7.48	7.53	7.53	7.50	7.62
7.52	7.55	7.75	7.75	7.56	7.56
6.43	6.43	6.46	6.40	6.44	
5.16		5.12	5.18	5.26	
6.89	6.89	6.83	6.89	6.78	6.88
6.66	6.71	6.67	6.73	6.60	6.72
4.72	4.72	4.61	4.72	4.70	4.87
14.4*	14.5	14.2	14.2	14.3	14.6
11.0*		10.9	10.9	10.8	10.9

[1] Dog urine.

Conceivably, hippuric acid and amino-acids might be sources of error. However, the first does not yield any nitrogen with hypobromite. Glycocoll yields about 3 per cent of its N. Inasmuch as the amount of amino-acids present in urine is very small to begin with[4] and provided that other amino-acids conduct themselves similarly to glycocoll, it is evident that the error from this direction is negligible. Creatine, which is sometimes a constituent of urine, liberates 2 of its 3 nitrogen atoms under the conditions of the experiment. Since it is usually absent or present only in small amounts this source of nitrogen may in general be neglected. Allantoin conducts itself very similarly to uric acid.

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[4] Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 301.

Table I contains some results obtained by the hypobromite method, and the urease method. In the case of the latter, determinations were made by Youngburg's modification as well as by the usual procedure of Van Slyke and Cullen.

In view of the general satisfaction given by the urease method the present method may seem superfluous. It is fitting, therefore, that its advantages be stated. They are: (1) Rapidity. Starting with a sample of urine the urea content may be known in 10 minutes. (2) Standard solutions are not employed. (3) Reagents necessary are simple and easily prepared. (4) There is practically no opportunity in the procedure for things to go awry as there are opportunities in the urease method. For example, the keeping qualities of dilute standard solutions and of urease solutions are not matters of concern. Neither is there any question of foaming nor about how long and at what rate to aerate.

Procedure.

25 cc. of diluted urine (diluted in the ratio of 1:10) are shaken with 4 gm. of permutit for 4 minutes. The mixture is then centrifuged or filtered. 1 cc. of the NH_3-free urine is introduced into the Van Slyke CO_2 apparatus, the last portion being rinsed in with 1 cc. of water followed by 1 cc. of sodium hypobromite solution. (This is made by mixing equal volumes of two solutions, one containing 12.5 gm. of sodium bromide and 12.5 gm. of bromide per 100 cc. and the other 28 gm. of sodium hydroxide per 100 cc.) The mercury is lowered to the 50 cc. mark and the apparatus is then shaken vigorously for about half a minute. The aqueous solution is collected in the proper chamber below the lower stop-cock, mercury is admitted to the 50 cc. chamber, and after adjusting the pressure the volume of nitrogen is read. Correction is then made for the dissolved air con-

tained in the diluted urine, the rinse water, and the hypobromite solution.[5]

It may be assumed with reasonable accuracy that the solubility of air in the diluted urine is the same as in pure water. For temperatures between 15 and 30°C. and a pressure of 1 atmosphere the solubilities are as given in Table II. The volumes are those which the gas would occupy at 760 mm. and the temperature in question and may, therefore, be subtracted from the gas volume as read. Determinations of the air dissolved in the hypobromite solution showed that between 15 and 20°C. this amounts to 0.006 cc. and between 21 and 25° to 0.005 cc.

TABLE II.

Cc. of Air Measured at 760 mm. of Mercury and the Temperature in Question per 1 Cc. of Water.

Temperature	Volume	Temperature.	Volume.	Temperature.	Volume.
°C.	cc.	°C.	cc.	°C.	cc.
15	0.0216	21	0.0198	27	0.0184
16	0.0212	22	0.0196	28	0.0182
17	0.0209	23	0.0193	29	0.0180
18	0.0206	24	0.0191	30	0.0178
19	0.0203	25	0.0188		
20	0.0201	26	0.0186		

The corrected volume is then reduced to standard conditions (0° and 760 mm. mercury) by means of the following formula.

$$V_o = V \frac{P_o - h}{(1 + 0.00367\,t)\,760}$$

where

V = gas volume as measured.
P_o = corrected barometric pressure.
h = aqueous tension.
t = temperature at which gas was measured.

To facilitate the calculation it will be found advantageous to refer to almost any of the compilations of physical and chemical data for useful gas reduction tables.

Once the volume of nitrogen measured at 0° and 760 mm. of Hg has been determined the weight is determined by multiplying by 0.0012507, the weight of 1 cc. of nitrogen. Taking the dilution and amount of sample into consideration the amount of urea nitrogen per 1 cc. of urine is easily found.

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[5] The correction for the air content of the diluted urine and rinse water can be eliminated by extracting the two in the apparatus and expelling the extracted gases before adding the hypobromite.

Some consideration was given to the adaptability of the hypobromite reaction to the determination of ammonium salts in urine as well as to the determination of urea. Theoretically there are no difficulties. The difference between the quantities of nitrogen liberated by diluted whole urine and by urine treated with permutit represents ammonia N and that only. The difficulty is in the measurement of the two quantities with sufficient accuracy to make it possible to express the ammonia N concentration with at least two significant figures. The ordinary Van Slyke apparatus cannot be read without a possible error of at least 0.003 of a cc. in the opinion of the writer. In addition no protection is provided against slight differences between the temperatures of the gas itself and the air registered by the thermometer close by. Consequently since the ammonia N is usually less than one-tenth of the sum of the ammonia and urea nitrogen and therefore occupies less than one-tenth of a cc. in the apparatus, its volume cannot be expressed accurately in thousandths of a cc. as is necessary if the result is to be expressible with two significant figures. If an approximate relation between the urea N and ammonia N is desired this can be very readily obtained.

SUMMARY.

A method for determining urea in urine is described which is both brief and accurate. Ammonium salts are removed by treating the urine with permutit and the ammonium-free solution is then subjected to the action of sodium hypobromite in the vacuum obtained with a Van Slyke CO_2 apparatus. Nitrogen is liberated quantitatively from the urea but to an entirely negligible extent from other urinary constituents.

Comparative analyses obtained with the urease and hypobromite procedures demonstrate the accuracy of the method.

FURTHER IMPROVEMENTS IN THE NEPHELOMETER-COLORIMETER.

By PHILIP ADOLPH KOBER and ROBERT E. KLETT.

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(Received for publication, March 29, 1921.)

The colorimeter described by Kober[1] has been improved in certain details which have resulted in the instrument described in the present paper. The first departure in construction from the Duboscq model was made by Kober[1] in 1915. It consisted of a 100 mm. scale, tiltable stand, adjustable verniers, fused black glass plungers, a rack, and a pinion. In 1917 this model was abandoned for the present one,[2] which has incorporated in it a fine screw arrangement in back of the instrument, away from possible contact with corroding liquids overflowing from the cups, in place of the rack and pinion exposed to such accidental overflowing by virtue of its position, retaining the fused black glass plungers with clear glass bottoms, but also having fused cups made of different colored glass which, in conjunction with the black plungers, made the transformation of the colorimeter into a nephelometer a matter of simply changing the cups. This 1917 model also possessed an ingenious device for adjusting the verniers to the zero point. Besides these changes there was added a set of split reflectors for the regulation and adjustment of light reflection. The hollow black plungers having fused optical ends, have been found to possess a special advantage in hydrogen ion determinations by Duggar.[3] He found that by putting shield solutions into the plungers as well as into the cups, a comparator for hydrogen ion and other work of great accuracy resulted. The split reflectors have also been found of special use by Field.[4] Where an unknown solution has a slight turbidity which absorbs light, thereby darkening the transmitted light, Field operates the reflector underneath the standard solution so that equal darkening is obtained.

Bock and Benedict[5] in 1918 described an instrument that is in reality a half plunger and a half cell instrument. This instrument has one advantage over most instruments of the plunger type; namely, that the scale and eyepiece can be viewed from one position of the observer, usually while sitting. The disadvantage of this type of instrument is the lack of symmetry or lack of interchangeability of the two sides of the instrument and of the two paths of light. Where only one or two heights of standard solution are necessary as in certain routine work this instrument is suitable, but this lack of flexibility in height of standard solution and the limited scale (40 mm.) make it less suitable for research and scientific work.

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[1] Kober, P. A., and Graves, S. S., *J. Ind. and Eng. Chem.*, 1915, vii, 843.

[2] Kober, P. A., *J. Biol. Chem.*, 1917, xxix, 155.

[3] Duggar, B. M., Annals of the Missouri Botanical Gardens, 1919, vi, 179.

[4] Field, C. W., personal communication.

[5] Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxv, 227.

In 1918–19 Leitz[6] made a decided change in construction in one of their colorimeters, by substituting a lever arrangement for the rack and pinion. This departure from the rack and pinion (Duboscq) or from the fine screw arrangement (Kober) which is inherent in accurate measuring devices, is obviously a step backwards in colorimeter construction. Another objection to levers is that they are apt to move from a fixed position through jars or accidental touch, no matter how slight.

In 1920 Bausch and Lomb[7] with the aid of Folin brought out a colorimeter of the Duboscq type but which has a number of changes from the French model. Four marked changes are apparent: (1) The base and upright frame are of very heavy castings, "to provide stability and permanent alignment of the optics" (2) The rack and pinion are so changed that the operating heads are always in a fixed position, so that the observer's readings are not influenced by the position of the pinion heads (3) The colorimeter cups used are of ground glass cylinders and plates, incased in heavy metal. (4) The adjustable verniers of the Kober instrument are adopted. The heavy metal and construction provide an instrument resistant to rough handling, and the fixed position of the milled heads seems to be an improvement over the French model, but the extra massiveness of the parts plays no rôle in its accuracy.

The instrument makers furnish attachments which make it possible to convert it into a nephelometer, after disconnecting plungers and adding these parts. This transformation while not a very convenient one, is of value. The means used to eliminate the meniscus from the nephelometric tubes or vials, and the care taken to eliminate the glare from the light source, which is designed to give parallel rays, are commendable features of this instrument.

During the routine tests of the Kober instruments it has become evident that the physical condition of the operator is an important factor in maintaining a high average of accuracy in adjusting the optical arrangement. It was found that frequent stooping down, in order to read the scale after having matched the colors or light in the eyepiece, induced fatigue very quickly. This stooping is necessary when using any of the Duboscq type instruments. This condition of fatigue was aggravated by the difference between the short focal distance in the eyepiece and the longer focus necessary to observe the scale, the result being greater eye fatigue.

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[6] Advertised by E. Leitz, in *Science*, 1919.

[7] Advertised by Bausch and Lomb, in *J. Ind. and Eng. Chem.*, 1920.

Another source of temporary fatigue which even if it is not so pronounced yet is a factor in accurate work, is the holding or supporting of the arms while adjusting or turning milled heads, to operate the plungers or cups. As long as the nephelometer-colorimeter was only used occasionally, these defects of course were not noticeable, but since their use has become daily and in many cases almost continuous, these factors have become important. Many experiments and models were made to eliminate the sources of annoyance and fatigue inherent in the Duboscq type, and although several models achieved the final result, the one described in this paper was adopted as the most satisfactory from all points of view especially since there is no indirect transmission in changing the vertical scale to a horizontal one.

The improvements, which eliminate these defects are: (1) The milled heads, formerly at the top of the instrument, are placed at the bottom, which allows the hands to rest on the table or other support and the adjustments to be made with the fingers (shown in Fig. 1). (2) An auxiliary scale is provided at the top of the instrument consisting of: two scales engraved upon the side away from the operator, fastened to the movable stages, so that when the stage is being moved up or down, the scales move with it; a stationary vernier, protruding beyond the top plate, also engraved upon the side away from the operator, fastened to the top of the instrument. A mirror facing the operator at an angle of 45° is placed in front of the protruding scale and vernier, so that an image of the two is reflected vertically. A magnifying glass of the same focal distance as the telescope, serving as a second eyepiece, has been placed close beside the regular eyepiece, directly above the mirror, showing the image of the scale enlarged in good light.

Fig. 1 shows the entire instrument without the lamp house.[8]

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[8] This instrument is manufactured by Klett Manufacturing Company, Inc., 202 East 46th Street, New York.

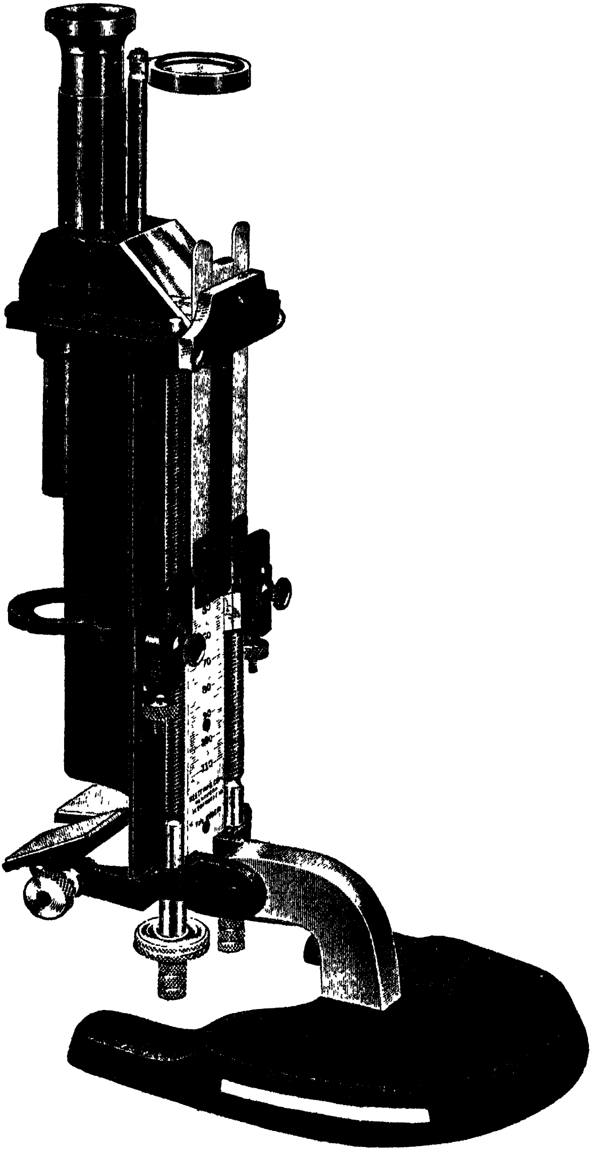

Fig. 1. View of the instrument without the lamp house, showing (a) the two eyepieces, the scale, and the mirror at the top of the instrument; (b) the micrometer adjustment of the zero point; (c) the milled heads operating the cups at the bottom of the instrument.

In Fig. 2 are shown the fields that are observed through the two eyepieces. The ease of reading the scale is apparent.

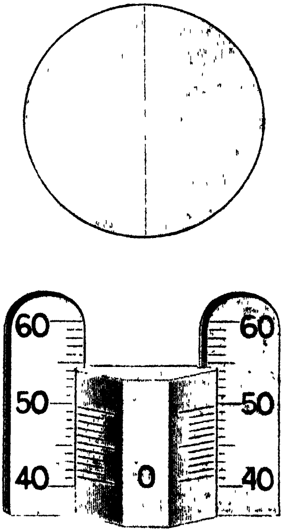

Fig. 2. The two fields as seen through the two eyepieces. The upper field shows the two semicircular fields; the lower field shows the stationary vernier and the two adjustable scales.

In Fig. 3 is shown the construction of the auxiliary scale. This auxiliary scale is engraved to 60 mm. but with the vernier is readable to only 50 mm., which is ample for most work. If heights above 50 mm. are to be measured the original vernier can be used. The setting of the zero point is easily and accurately accomplished with a micrometer arrangement, as may be seen at A by a milled head working against a spring. This convenient method of zero point adjustment, together with the very simple method of using the instrument, the method of Lamb, Carleton, and Meldrum,[9] where the height of the standard solution (S) is kept constant, makes the operation of the instrument and the calculation of results extremely simple and easy without, however, sacrificing accuracy or deviating from the fundamental basis of either colorimetry or nephelometry.

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[9] Lamb, A. B., Carleton, P. W., and Meldrum, W. B., *J. Am. Chem. Soc.*, 1920, xlii, 252.

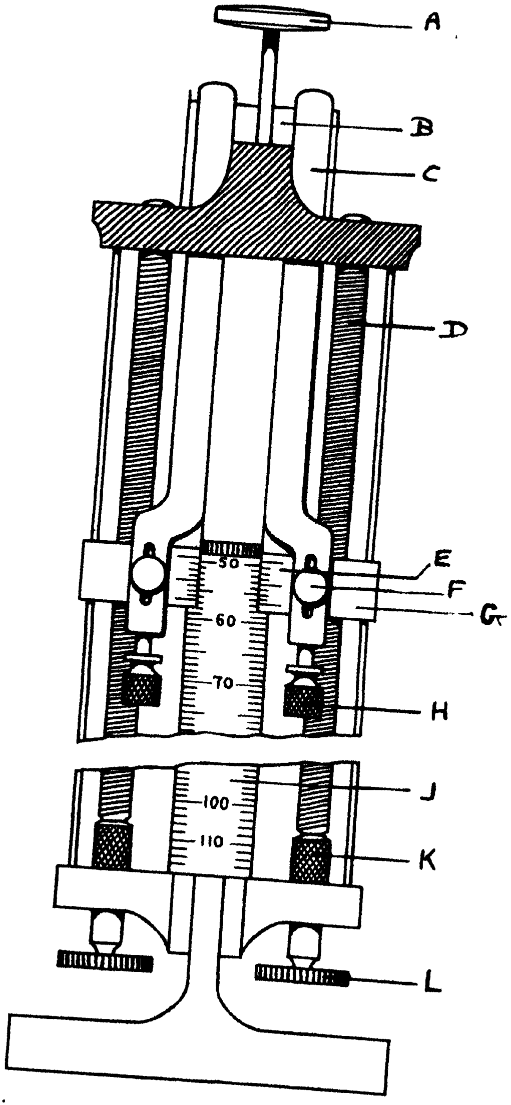

Fig. 3. Diagrammatic sketch of the rear of the instrument. A, magnifying lens for the horizontal scale; B, mirror at an angle of 45°; C, movable scale; D, screw-threaded rod; E, vernier for 50 to 100 mm. scale; F, lock nut for the zero adjustment; G, movable cup carrier; H, micrometer for zero adjustment; J, scale from 50 to 100 mm.; K, knurled thumb screw for rapid movement; L, knurled thumb screw for fine adjustment.

In Fig. 4 is shown the instrument attached to a lamp house.

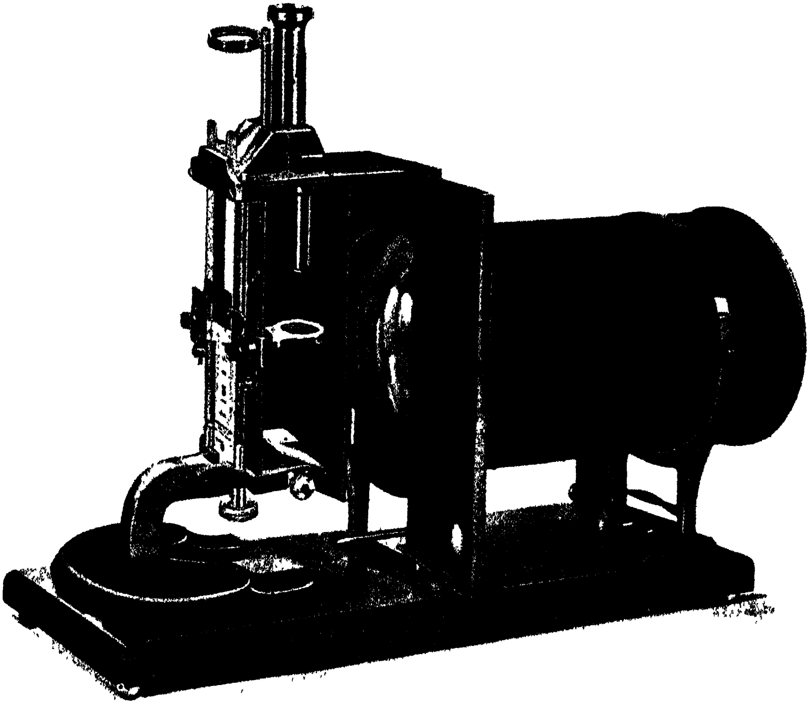

FIG. 4. The instrument and lamp house showing the split reflectors as well as the front of the instrument illuminated by the light from the lamp house.

SUMMARY.

The advantages of the new improvements are: (1) the elimination of the fatigue and annoyance, due to stooping to read the scale of Duboscq instruments; (2) an enlarged and well illuminated scale read through an eyepiece of the same focal length as the telescope; (3) a more convenient position for the milled heads operating the stages, allowing for resting of the operator's arms; and (4) a micrometer arrangement for setting the zero point which can be locked in any position.

ON THE SUBSTITUTION OF TURBIDIMETRY FOR NEPHELOMETRY IN CERTAIN BIOCHEMICAL METHODS OF ANALYSIS.

By W. DENIS.

(From the Laboratory of Physiological Chemistry, Tulane University Medical School, New Orleans.)

(Received for publication, April 25, 1921.)

Within the past decade nephelometric methods of analysis have become increasingly popular in biochemical work, so that at present a nephelometer has come to be counted an indispensable piece of apparatus in every well equipped laboratory. Nephelometry has however the serious disadvantage that, as was first pointed out by Richards and Wells,[1] the amount of light reflected is not strictly proportional to the weight of the precipitate under observation, but seems to be influenced by a variety of factors. To overcome this defect Richards and Wells adjusted the volume of their solutions so that standard and unknown contained about the same concentration. Kober[2] has suggested a mathematical formula by means of which he has obtained excellent results, and Bloor[3] recommends that a standard be selected which is of such strength that it varies not more than 25 per cent from the unknown.

During the past 5 years the author has had occasion, in connection with studies on blood and milk, to make extensive use of nephelometric methods, and as a result of this experience has come to appreciate more clearly the relatively large error which may be introduced in an analysis unless the strength of the standard and of the unknown are adjusted to within at least 10 per cent, a requirement which means that for a single determination it may be necessary to provide from three to six standards of varying degrees of concentration. The irksomeness of preparing large numbers of standards has led to an investigation concerning the possibility of utilizing the turbidimeter in place of the nephelometer in several biochemical methods of analysis.

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[1] Richards, T. W., and Wells, R. C., *Am. Chem. J.*, 1904, xxxi, 235.

[2] Kober, P. A., *J. Biol. Chem.*, 1912-13, xiii, 485.

[3] Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.

Turbidimetric methods of analysis are extensively used in technical work, as for example in connection with the determination of suspended matter in water, of sulfur in coal, etc., but so far but little use has been made of this principle in the solution of biochemical problems. Some years ago Folin and Denis[4] made use of this technique for the determination of albumin in urine, and recently Denis and Ayer[5] have employed a similar method in the analysis of cerebrospinal fluid. In connection with the above work, and as the result of a series of readings made on standard solutions of widely varying concentrations we felt justified in believing that turbidity determinations made on the precipitate obtained by the action of sulfosalicylic acid on protein give comparable results even when the concentration of the unknown and of the standard vary as much as 50 per cent. If it could be proved that this finding also applies to the measurement of suspensions other than protein, the usefulness of the turbidimeter in biochemical work becomes immediately apparent.

Although several types of turbidimeters have been suggested, I have continued the use of the Duboscq colorimeter for the measurement of my suspensions. To obtain the best results all readings should be made in a dark or semidark room, and as a preliminary to any series of readings the position of the colorimeter should be so adjusted with relation to the source of light, that exactly the same illumination is obtained on both sides when both cups are filled with the standard suspension and both scales are set at the same point. It has been my experience that the most accurate results are secured when the mirror is adjusted to give the maximum illumination.

In this paper I wish to present the results of a study which has for its basis the attempt to substitute turbidimetry for nephelometry in three analytical procedures; *viz.*, the determination of calcium in blood by Lyman's method[6] (in which the blood calcium in the form of colloidal calcium stearate is measured by comparison with a standard calcium stearate suspension); the determination of fat in blood and milk by the method of Bloor[7] (which involves the use of colloidal suspensions of fatty acids); and of phosphates by the strychnine molybdate precipitate of Pouget and Chouchak[8] as modified by Kober and Egerer[9] and by Bloor.[3]

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[4] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 273.

[5] Denis, W., and Ayer, J. B., *Arch. Int. Med.*, 1920, xxvi, 436.

The results obtained with calcium stearate suspensions indicate that it is easily possible to obtain quantitative results with suspensions varying in concentration by as much as 50 per cent, provided these suspensions are of such concentration that they contain between 0.75 and 0.35 mg. of calcium in a final volume of 100 cc.; with greater concentration precipitation frequently occurs; while if the amount of calcium is less than 0.35 mg. per 100 cc. turbidimetric readings can no longer be made with precision.

TABLE I.

Comparison of Results Obtained by the Use of the Nephelometer and the Colorimeter in the Determination of Calcium in Milk.

Observation No.	Ca per 100 cc. of milk.	
	By nephelometry.	By turbidimetry.
	mg.	*mg.*
210	18.6	18.6
237	22.1	22.9
239	20.0	20.0
235	19.2	19.4
240	33.3	33.1
269	21.6	21.0
232	17.2	17.4

In Table 1 are presented the results obtained in a series of determinations of calcium in milk by Lyman's method in which parallel readings were made by the turbidimeter and by the nephelometer.

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[6] Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.

[7] Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.

[8] Pouget, I., and Chouchak, D., *Bull. Soc. chim.*, 1909, v, series 4, 104.

[9] Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

With fatty acid suspensions quantitative readings can be made when the standard and unknown vary by 60 per cent provided the concentrations of fatty acid lie within the range of 8 to 2 mg. per 100 cc.

In Table II comparison is made of analyses of milk fat made by the Babcock method, and by Bloor's method, readings having been made by means of the turbidimeter instead of the nephelometer.

TABLE II.

Comparison of Results Obtained by Determinations of Fat in Milk by the Babcock Procedure and by the Use of the Colorimeter in Bloor's Micro Fat Method.

Observation No.	Fat	
	By Babcock method.	By turbidity
	per cent	*per cent*
1	3.2	3.2
2	3.1	3.0
3	3.5	3.6
4	4.3	4.4
5	4.7	4.4
6	5.0	5.1

TABLE III.

Comparison of Results Obtained by the Use of the Nephelometer and the Colorimeter in the Determination of Inorganic Phosphates in Blood Plasma.

Observation No.	P per 100 cc of plasma.	
	By nephelometer.	By colorimeter.
	mg.	*mg.*
143	2.3	2.2
144	2.6	2.7
163	3.0	3.0
162	3.8	2.6
160	5.0	5.9
247	6.2	6.0

Results, essentially similar to those reported above, were obtained with strychnine phosphomolybdate suspensions. The optimum concentrations for turbidimetric work with this material were found to be 0.12 to 1.2 mg. H_3PO_4 per 100 cc.

In Table III are collected the results of a series of determinations of the inorganic phosphates of blood plasma in which parallel readings were made by the nephelometer and by the turbidimeter.

SUMMARY.

The suggestion is made that determinations of turbidity, made by means of a colorimeter, may with advantage be substituted for nephelometric readings in several analytical processes. The advantages of this procedure are twofold: first, as turbidimetric readings give quantitative results with large variations in concentration between standard and unknown it is possible to omit the preparation of the large number of standards which have been found necessary in nephelometric work; second, it presents an additional use for the now universally owned colorimeter and in the case of many small laboratories would probably make the possession of a nephelometer unnecessary.

CREATINURIA.

II. ARGININE AND CYSTINE AS PRECURSORS OF CREATINE.*

BY E. G. GROSS AND H. STEENBOCK.

(From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.)

(Received for publication, April 20, 1921.)

In a previous publication (1) we have analyzed the various factors which may be operative in the production and prevention of creatinuria. This analysis was suggested by the possibility that the anomalies observed in the appearance of creatinuria could very well owe their causation to the variation in the utilization of amino-acid precursors of creatine with different physiological states or functions. Processes of growth, fetus building, and lactation would have their influence on creatinuria in modifying the intake and utilization of amino-acids and therefore varying the residue left to be catabolized into creatine. Creatine excretion could thus be indicated, not only as a result of excessive ingestion of its precursors, but also as a result of deficient ingestion of the complements of the precursors necessary to make protein building possible. Excessive catabolism such as obtains during fasting, fever, acidosis, and phosphorus poisoning would then similarily owe its creatinuria to the excessive liberation of creatine precursors although in part some of the creatine excreted would originate from the creatine normally found as a constituent of muscle.

Our previous investigations designed to correlate some of these factors have shown that creatinuria can always be induced in the pig by feeding sufficient protein. This was found to be the case irrespective of the sex of the animal, or the acidity or alkalinity of the diet. As a result we have continued our study of the problem in an attempt to define further its origin.

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* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

In the search for possible precursors of creatine, arginine has attracted the most attention because, like creatine, it is a derivative of guanidine and because it is contained in all the known proteins. In fact it is the only cleavage product of protein so far isolated which contains the guanidine nucleus.

While it is now generally accepted that creatine is derived from proteins, such a difference of opinion exists in the literature as to the possibility of arginine being the precursor of creatine, that no definite conclusion as to the exact state of affairs can be drawn. Direct experimentation with the feeding of arginine has been rather limited but considerable work has been done by transfusion and injection. Inouye (2) found an increase in creatine upon adding arginine to liver extract, and also when arginine was perfused through the surviving liver. Myers and Fine (3) in feeding rats with edestin, which contains 14 per cent of arginine, and casein, which contains 4 per cent of arginine, found a slight increase in the muscle creatine with the former, but they draw no conclusions. Thompson (4) reported an increase in urinary creatine following injection of arginine carbonate, but failed to obtain an increase by oral administration. Jaffé (5) found no increase in urinary creatine following the feeding of arginine to the rabbit.

Recently Harding and Young (6) have suggested that cystine might also be metabolized into creatine "through the intermediate stages of taurine and amino ethyl alcohol, followed by methylation, combination with urea, and oxidation." Using dogs for the experiments they obtained increases with cystine but not with arginine. It appeared significant to us that in their report they state that the endogenous creatine was affected as well as the exogenous. This suggested that possibly what was interpreted by them as a direct affect of cystine was in reality an indirect effect—the cystine stimulating catabolism by means of the sulfuric acid formed by its oxidation. Of their failure with arginine we will have more to say later.

In this paper there will be presented data showing how arginine and cystine may function in inducing or augmenting creatinuria.

EXPERIMENTAL.

In the following experiments as in the past, the pig was chosen as the experimental animal as it stands confinement well and can be fed a nitrogen-free diet over a long period of time. Such a diet offers especial advantages in that it simplifies the conditions of experimentation and allows more accurate interpretation of the data. Methods of analysis and the experimental procedure used were the same as before (1). Total nitrogen and creatinine determinations were made to serve as an index of general excretory activity.

The arginine used was prepared according to Kossel's method from the protein arachin. As a check on its purity it was analyzed according to Van Slyke. To remove traces of barium which always tend to be carried along in the preparation, sodium sulfate was added in excess. In later experiments, Tables IV and V, however, the barium was removed quantitatively with sulfuric acid as it was feared that the sodium carbonate formed in the other precedure might affect the results.

The arginine was usually given by stomach tube. In the experiments of Table IX it was given in gelatin capsules. Usually some difficulty was experienced in getting the pig to retain it as it appeared to be nauseating. Some success was had in neutralizing or acidifying it with acetic or lactic acid but even with this precaution the largest amount that could be given without always causing vomiting or diarrhea was the equivalent of 100 gm. of casein.

The cystine given was prepared from human hair by acid hydrolysis. Analyses showed it to contain 26.63 per cent sulfur, the theoretical being 26.69 per cent. It was administered in water suspension by stomach tube.

Not all the pigs were found suitable for the demonstration of the effect of these amino-acids. In some the production of creatine was so low that excessive casein, 300 to 400 gm. had to be fed before the threshold for creatine excretion was reached. As the arginine equivalent of such amounts of casein could not be successfully given these animals were discarded. With all pigs, preliminary to the amino-acid administrations, casein was given for purposes of comparison as to the amounts of creatine produced.

Experiments with Arginine.

TABLE I.

Pig, male, weight 28 kilos. Energy intake, 75 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*	*gm.*	*gm.*	
11	1,550	4.40*	0.857	0.068	Starch 500 gm.
12	1,400	2.57	0.945	0.044	" 500 "
13	1,225	3.62	0.772	0.168	" 500 " arginine 4.08 gm.
14	1,575	2.52	0.849	0.000	" 500 "
15	1,300	2.10	0.819	0.084	" 500 "
16	1,450	2.32	0.804	0.073	" 500 "

* The high urinary nitrogen represents in part residual nitrogen from an attempt to feed 200 gm. of casein 2 days previously.

In Table 1 is shown the effect of arginine on creatine production. Previous to the collection of the data here presented, observations had been taken on the effect of casein administration. When 100 gm. of casein were given, after 4 days maintenance on a starch diet, the creatine increased from an average value of 60 to 103 mg. An attempt to further increase the excretion by giving 200 gm. of casein resulted in partial anorexia. The nitrogen excretion on the first day bears evidence, however, as shown in the table, that the casein had been partially consumed.

TABLE II.

Pig, female, weight 24 kilos. Energy intake, 75 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*	*gm.*	*gm.*	
8	1,450	1.94	0.460	0.053	Starch 400 gm.
9	2,200	2.20	0.344	0.073	" 400 "
10	1,200	5.54	0.342	0.163	" 300 " casein 100 gm.
11	1,200	2.80	0.330	0.065	" 400 "
12	1,250	1.85	0.485	0.075	" 400 "
13	1,450	2.92	0.468	0.087	" 400 " arginine 4.08 gm.
14	1,550	1.92	0.451	0.121	" 400 "
15	1,650	1.88	0.478	0.051	" 400 "
16	1,500	1.68	0.505	0.085	" 400 "

In Table II is brought out the effect of casein and arginine in creatine production. In both cases the creatine excretion was increased, although after arginine feeding, the increase was not observed until the second day. This retardation in excretion has also been noted after casein feeding, even though the major portion of the nitrogen was excreted on the first day. We believe this is indicative of the fact that we are dealing with a special form of amino-acid metabolism which is independent, at least to a considerable extent, of the major processes of protein metabolism.

TABLE III.

Pig, male, weight 25 kilos. Energy intake, 65 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Diet.	
	cc.	*gm.*	*gm.*	*gm.*		
20	1,850	1.59	0.410	0.121	Starch 400 gm.	
21	1,800	1.33	0.427	0.090	" 400 "	
22	1,850	3.95	0.415	0.238	" 300 "	casein 100 gm.
23	1,800	2.48	0.382	0.122	" 400 "	
24	2,000	1.76	0.384	0.112	" 400 "	
25	1,750	3.11	0.398	0.162	" 400 "	arginine 4.08 gm.
26	1,800	1.40	0.382	0.122	" 400 "	
27	2,000	1.52	0.368	0.100	" 400 "	
28	1,850	3.14	0.415	0.278	" 300 "	casein 100 gm.
29	2,350	2.39	0.305	0.103	" 400 "	
30	1,850	1.36	0.360	0.125	" 400 "	

Table III again shows a distinct, though small, rise in creatine excretion after arginine administration. It is not comparable to the rise obtained on casein of equal arginine content. In fact, this is what is generally observed. The arginine given in this experiment was neutralized with lactic acid, instead of acetic acid, after sodium sulfate had been added to remove the barium.

Tables IV and V also bear testimony to the fact that arginine as well as casein can increase the creatinuria. These tables are presented as examples of what has been obtained time and again, none of them showing any great uniformity in creatine production. Sometimes a rise of 30 mg. was obtained, then again one of 115 mg., and occasionally none at all. This latter observation is not to be taken as one which invalidates our contention that arginine is a precursor of creatine as in such instances a drop in the excretion independent of dietary influences may have occurred and the arginine administered merely counterbalanced this decrement. Such changes, irrespective of dietary modifi-

TABLE IV.

Pig, male, weight 28 kilos. Energy intake, 75 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Diet.	
	cc.	gm.	gm.	gm.		
5	1,600	1.76	0.507	0.052	Starch 500 gm.	
6	1,800	1.43	0.534	0.036	" 500 "	
7	1,750	1.57	0.585	0.055	" 500 "	
8	2,000	2.25	0.595	0.045	" 500 "	
9	1,550	3.13	0.541	0.192	" 400 "	casein 105 gm.*
10	2,000	2.40	0.695	0.115	" 500 "	
11	2,100	1.34	0.598	0.086	" 500 "	
12	1,850	2.59	0.654	0.154	" 500 "	arginine 4.08 gm.
13	1,850	1.83	0.675	0.058	" 500 "	

* 105 gm. of this casein was required to furnish the same amount of nitrogen as 100 gm. before.

TABLE V.

Pig, male, weight 29 kilos. Energy intake, 70 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen	Preformed creatinine.	Creatine as creatinine	Diet	
	cc	gm	gm.	gm		
9	2,100	1.47	0.430	0.042	Starch 500 gm.	
10	2,000	1.72	0.505	0.070	" 500 "	
11	2,200	2.44	0.533	0.182	" 400 "	casein 105 gm.
12	1,650	2.31	0.539	0.000	" 500 "	
13	1,700	2.72	0.489	0.131	" 500 "	arginine 4.08 gm.
14	2,300	2.34	0.517	0.195	" 500 "	
15	2,700	1.72	0.561	0.060	" 500 "	
16	2,200	1.64	0.616	0.066	" 500 "	
17	2,500	2.20	0.415	0.205	" 500 "	arginine 4.08 gm.
18	2,400	1.92	0.479	0.143	" 500 "	
19	1,800	1.76	0.468	0.072	" 500 "	

cations, are very common, in fact, in some pigs the creatinuria may disappear entirely only to reappear upon the administration of very small amounts of protein. Apparently the production of creatine in these cases has merely fallen below the threshold of its excretion.

As a general occurrence, more creatine was excreted after casein feeding than after the administration of its arginine equivalent—the former exceeding the latter by about 25 mg. or 25 per cent. This is hardly surprising when we take into consideration the fact, that acids, such as phosphoric acid which is liberated in the metabolism of casein, stimulate the production of creatinuria, but two other possibilities also suggest themselves. In the first place, the casein molecule may carry still other precursors of creatine to augment its excretion, and in the second place, it is possible that free arginine may be metabolized to urea faster—and thus circumvent its formation into creatine—than the arginine as absorbed with the products of digestion. These are mere hypotheses of which only the first one can derive any support from facts now available.

Experiments with Cystine.

In the studies of the production or augmentation of creatinuria by cystine feeding, analyses of the urines for total sulfur and sulfates as well as for total nitrogen, creatine, and creatinine were made. Total sulfur was determined by Benedict's method and total sulfates by Folin's method. 4.08 gm. of cystine—the same amount as arginine previously—were given to the pig in each trial.

In Tables VI and VII it is seen that cystine feeding increased the creatinuria to about the same degree as, or possibly slightly more, than the same amount of arginine. In Table VI after the first cystine administration the rise in creatine was delayed 1 day. This is similar to what we have seen with arginine and possibly indicates that the creatine is subject to special laws of excretion in comparison with the total nitrogen, sulfur, and other compounds. The cystine sulfur makes its appearance in the urine vary largely in the oxidized form, showing a tendency to a disturbance of the acid-base balance. Acidity determinations were not made.

TABLE VI.

This record is a continuation of that shown in Table IV, the same pig being used throughout. 500 gm. of starch were fed daily.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Dietary addition.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
14	2,200	1.27	0.533	0.094	0.475	0.255	0.220	
15	2,200	1.45	0.550	0.027	0.446	0.279	0.166	
16	1,800	1.40	0.575	0.052	0.399	0.234	0.162	
17	2,450	2.53	0.587	0.038	1.029	0.718	0.310	Cystine 4.08 gm.
18	2,150	1.41	0.596	0.150	0.627	0.462	0.165	
19	1,950	1.48	0.472	0.039	0.764	0.580	0.184	
20	1,950	2.34	0.589	0.087	0.593	0.378	0.215	
21	2,300	2.85	0.644	0.132	1.110	0.668	0.442	Cystine 4.08 gm.
22	2,150	1.72	0.612	0.039	0.602	0.385	0.217	
23	1,950	1.82	0.560	0.062				

TABLE VII.

This record is a continuation of that shown in Table V, 3 days' collection being omitted. 500 gm. of starch were fed daily.

Day of starch feeding.	Volume of urine	Urinary nitrogen.	Preformed creatinine	Creatine as creatinine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Dietary addition.
	cc	*gm*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
23	2,100	1.72	0.582	0.053	0.359	0.310	0.048	
24	2,100	1.93	0.619	0.063	0.462	0.277	0.184	
25	2,150	2.66	0.541	0.155	0.673	0.364	0.309	Cystine 4.08 gm.
26	2,200	2.24	0.632	0.083	0.712	0.495	0.217	
27	2,000	2.04	0.650	0.100	0.535	0.351	0.184	
28	2,200	1.76	0.577	0.066	0.485	0.323	0.162	
29	2,150	2.15	0.617	0.129	0.939	0.558	0.384	Cystine 4.08 gm.
30	2,400	2.18	0.538	0.089	0.683			
31	2,300	1.93	0.542	0.058	0.618	0.367	0.251	

Table VIII shows how creatinuria is influenced by acidosis. Normally on a starch diet the urine of a pig is always acid. Under such conditions, with this particular animal creatine was always excreted. When the urine was changed to an alkaline reaction by sodium acetate administration the creatine promptly disappeared. Under these conditions cystine feeding was without effect in each of the four individual trials attempted; casein, on the other hand, was effective as usual. This seems to prove beyond a question that cystine creatinuria owes its origin to other factors than those operative when casein is fed.

TABLE VIII.

Pig, male, weight 40 kilos. Energy intake in the form of starch, 75 calories per kilo except when casein was given; then an equivalent isodynamic reduction in starch intake was made.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Total sulfur.	Total sulfate sulfur.	Neutral sulfur.	Dietary addition.
	cc.	*gm.*	*gm.*	*gm.*	*gm*	*gm.*	*gm.*	
14	2,300	2.25	1.177	0.101				
15	1,050	1.98	1.344	0.115	0.424	0 329	0.095	Sodium acetate 50 gm.
16	1,000	2.32	1.470	0.000	0.508			“ “ 50 “
17	2,000	2.72	1.200	0.000	0.560	0.320	0.240	“ “ 50 “
18	2,250	2 92	1.327	0.000	1.417	0.732	0.685	“ “ 50 “ cystine 4.08 gm.
19	2,150	2.27	1.128	0.000	0.691	0.645	0.046	Sodium acetate 20 gm.
20	1,750	5.32	1.235	0.222	0.937	0.576	0.361	“ “ 50 “ casein 165 gm.
21	1,800	2.11	1.151	0.000	0.335	0.343	0.191	Sodium acetate 25 gm.
22	2,800	1.48	1.109	0.000	0.806	0.761	0.045	“ “ 25 “
23	2,800	3.81	1.299	0.000	0.576	0.375	0.201	“ “ 50 “ cystine 4.08 gm.
24	2,700	2.43	1.296	0.000	0.475	0.278	0.197	Sodium acetate 25 gm.
25	2,100	2.56	1.207	0.000	1.032	0.777	0.265	“ “ 50 “ cystine 4.08 gm.
26	3,100	2.70	1.010	0.000	0.750	0.540	0.210	Sodium acetate 25 gm.
27	2,400	2.44	1.190	0.000	1.190	1.040	0.150	“ “ 50 “ cystine 4.08 gm.
28	3,000	2.10	1.080	0.000	0.422	0.308	0.014	Sodium acetate 25 gm.
29	1,800	5.18	1.212	0.168	0.765	0.582	0.183	“ “ 50 “ casein 165 gm.
30	3,000	6.06	1.033	0.080	0.525	0.315	0.210	Sodium acetate 25 gm.

TABLE IX.

A continuation of Table VIII with the same dietary régime but with other additions, comparing the effect of arginine with cystine administrations on creatinuria production.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	gm	gm.	gm.	
31	2,150	3.27	1.131	0.000	Sodium acetate 25 gm.
32	2,150	3.39	1.139	0.140	" " 25 " arginine 4.08 gm.
33	2,950	3.00	1.130	0.000	Sodium acetate 25 gm.
34	2,500	3.50	1.060	0.090	" " 25 " arginine 4.08 gm.
35	3,000	3.12	1.110	0.040	Sodium acetate 25 gm.
36	3,100	3.03	1.187	0.000	" " 25 "
37	3,600	2.52	1.034	0.000	" " 50 " cystine 4.08 gm.
38	3,850	3.08	1.155	0.000	
39	3,000	2.64	1.010	0.000*	
40	3,050	2.44	1.097	0.000†	
41	2,800	2.63	1.065	0.071‡	
42	3,400	2.65	1.144	0.067	
43	3,300	2.64	1.056	0.090	
44	3,500	3.57	1.038	0.160	Cystine 4.08 gm.
45	2,200	2.11	0.990	0.000	

* The urine, since the first administration of sodium acetate (Table VIII) had always been kept alkaline to phenolphthalein. On this day with the cessation of sodium acetate feeding it was acid to phenolphthalein but alkaline to litmus.

† Urine was neutral to litmus.

‡ Urine was acid to litmus on this day and subsequently.

The relations brought out in Table VIII are emphasized in their significance by the data presented in Table IX where creatinuria, when induced by arginine feeding is also shown not to be affected by sodium acetate administration. Under such conditions cystine was again shown to be unable to produce creatinuria as long as the urine remained alkaline. After the urine had become acid to litmus again creatine reappeared and increased in amount with cystine administration. Failure of its continued appearance is left unexplained as the experiment was terminated, but probably represents one of the variations in creatine excretion called attention to before.

SUMMARY.

Arginine administered orally in sufficient amounts augments creatine excretion in the pig.

Creatinuria induced by casein feeding appears to have its origin in large part in the formation of creatine from arginine, but the acidity of the phosphoric acid split off no doubt also contributes to the creatinuria as a result of the stimulation of endogenous metabolism.

Cystine feeding causes creatinuria only when the sulfuric acid formed by the oxidation of its sulfur is left unneutralized; when neutralized the creatinuria promptly disappears.

Neutralization of acidity does not prevent the creatinuria called forth by casein or arginine feeding.

BIBLIOGRAPHY.

1. Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265.
2. Inouye, K., *Z. physiol. Chem.*, 1912, lxxxi, 71.
3. Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 389.
4. Thompson, W. H., *J. Physiol.*, 1917, li, 111.
5. Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.
6. Harding, V. J., and Young, E. G., *J. Biol. Chem.*, 1920, xli, p. xxxvi.

CREATINURIA.

III. THE EFFECT OF THYROID FEEDING UPON CREATINURIA.*

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The study of any reaction in the animal body, where only the excretory end-products are available to indicate the course of the whole, is bound to reveal many apparent inconsistencies. Thus in attributing the origin of creatine to certain processes of protein metabolism, investigators are confronted with the undeniable fact that the most drastic attempts to induce creatinuria in men by protein feeding have met with failure. With women no such difficulty has been encountered. In our experiments (1) carried out with the pig no difficulty in the production of creatinuria by protein feeding has manifested itself, irrespective of the sex of the animal. We have, however, observed certain variations among individuals. For instance, some pigs do not excrete creatine in their urine when fed a starch diet or when on a normal ration, but when fasted for a number of weeks such individuals may or may not show the presence of creatine. We have also observed a great variation in the amount of casein necessary to produce creatinuria in the animals. Some require 100 gm., and others require as much as 300 gm. or more. Differences in behavior might be taken as an index of the nearness of approach of endogenous creatine production—though the exogenous would present similar relations—to the threshold level of its excretion. Yet even this does not tell the whole story as animals already showing creatinuria on a nitrogen-free diet often require more than 100 gm. of casein before any demonstrable increase in creatinuria results. Creatine precursors of exogenous origin evidently are submitted to a different array of metabolic processes from those originating endogenously. Otherwise, very

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small amounts of casein should suffice to increase a creatinuria already in existence. As a matter of fact Thompson (2) actually found that while he was unable to increase creatinuria in certain dogs by feeding arginine, upon injecting it subcutaneously 9 per cent was recoverable as creatine. Normally we believe that the great bulk of creatine formed is metabolized into creatinine and thus kept from accumulating in the blood stream to the level at which its excretion would become possible, but as this reaction is relatively constant among individuals and is not modified except by the administration of tremendous amounts of creatine it undoubtedly is a factor of little importance in the problem under consideration. The main possibility demanding our attention is the fact that creatine is formed or not formed in direct proportion to the balance that obtains between the arginase system, destructive as far as creatine formation is concerned, and the oxidative system whereby the guanidine grouping is left intact. A change in the velocity of either would immediately affect the end-result.

Under ordinary conditions the arginase reaction appears to be very prominent as the administration of arginine results in creatine formation to the extent of only 3 or 4 per cent of the theoretical possibility as shown in our experiments (3). Of the nature of the other mechanism practically nothing is known.

Denis (4) in 1917 reported the production of creatinuria, in a man afflicted with Graves disease, by protein feeding. As it is well known that in this malady the rate of metabolism is enormously increased it suggested itself to us that possibly the thyroid mechanism might be responsible for the differences in the result of protein feeding to normal men as compared with women. As the active principle, thyroxin, of the thyroid gland functions in oxidative reactions it appears possible that it may take part in influencing creatinuria in oxidizing arginine, thus removing it from the sphere of activity of arginase and increasing the amount of creatine formed. On the basis of these hypotheses an attempt was made to study the effect of feeding thyroid preparations on creatinuria of both exogenous and endogenous origin.

While a vast amount of information, relative to oxygen consumption and carbon dioxide production under thyroid influences, has been collected, surprisingly little work appears to have been done on the effect of thyroid secretion or preparations on nitrogen metabolism beyond establishing an increased nitrogen excretion. This has been observed by Magnus-Levy (5) and Andersson and Bergman (6) on feeding thyroid to normal men. Schöndorff (7), Gluzinski and Lemberger (8), and Voit (9) observed it with dogs and Farrant (10) with cats and rabbits. Underhill and Saiki (11) found but a slight increase in urinary nitrogen as a result of thyroid feeding with no change in urinary nitrogen distribution. Cramer and Krause (12), on the other hand, obtained an increased creatinuria with both men and dogs as the result of artificially induced hyperthyroidism.

TABLE I.

Pig, male, weight 31.5 kilos. Energy intake as starch, 65 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	*gm.*	*gm.*	*gm*	
5	1,650	3.63	1.012	0.033	
6	1,775	2.48	0.918	0.047	1 gm. thyroid.
7	1,800	2.66	0.990	0.084	1 " "
8	1,800	2.91	1.023	0.324	3 " "
9	1,650	2.57	0.957	0.264	
10	1,450	2.95	1.050	0.058	

EXPERIMENTAL.

In the following experiments desiccated sheep's thyroid prepared by Armour and Company was given to pigs after their nitrogen metabolism had been reduced to the endogenous type by starch feeding. The sheep's thyroid contained 0.14 per cent of iodine. It was administered, suspended in water, by stomach tube. Analyses for total nitrogen creatine and creatinine were made daily.

The data in Table I show that small amounts of thyroid are ineffective in changing the nitrogen metabolism. Not until 3 gm. were given was the creatine excretion increased. As the preparation contained only 3.6 mg. of creatine per gm. the increase could not be accounted for as being derived from exogenous sources. It is significant that the total nitrogen and creatinine remained constant as far as our technique allowed us to determine.

Tables II and III show the discrepancy that obtains between the creatine excretion induced by thyroid feeding and the increase in total nitrogen. Creatinine excretion was not increased which may not be so evident from Table III alone but becomes evident when the creatinine level shown here is compared with that shown on the same animal in Table IV. This again emphasizes the marked limitation of the animals ability to change creatine into creatinine, otherwise with the large increase in creatine to the point where it even exceeds the creatinine excretion, the latter would also have been increased.

TABLE II.

Pig, male, weight 28 kilos. Energy intake as starch, 74 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	*gm.*	*gm.*	*gm.*	
7	1,450	2.37	0.449	0.142	
8	1,400	2.88	0.488	0.150	4 gm. thyroid.
9	1,700	3.29	0.441	0.456	
10	1,200	2.49	0.393	0.376	
11	1,300	2.13	0.421	0.105	

TABLE III.

Pig, male, weight 29 kilos. Energy intake as starch, 70 calories per kilo. This record is a continuation of that shown in Table IV.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary additions.
	cc.	*gm.*	*gm.*	*gm.*	
21	1,500	1.65	0.702	0.160	
22	1,500	1.89	0.727	0.165	4 gm. thyroid.
23	1,700	2.89	0.680	0.707	
24	1,550	2.35	0.737	0.707	
25	1,500	2.37	0.630	0.412	
26	1,500	1.50	0.588	0.169	

While the total nitrogen increase does not appear to be commensurate with the creatine increase, which amounts to a three- to fourfold miltiplication when compared with total creatinine, comparisons appear warranted.

In Table II the total creatinine nitrogen ranges approximately from 8 to 11 per cent of the total nitrogen and in Table III from 16 to 22 per cent of the total nitrogen on different days. If these values could be accepted as indicating a relative constancy of relations in the individual it would lend support to our hypotheses that creatinine is formed from creatine, both having a common origin. If on the other hand they are to be considered inconstant it would tend to prove that thyroid medication exerted a special influence on the direction of protein catabolism in relation to the guanidine nucleus. Our data are hardly extensive enough to warrant either conclusion especially as we know that the urinary constituents obey different laws of excretion (13) and therefore changes in nitrogen distribution subsequent to thyroid administration for only 1 day could be expected to reveal little of importance in these guanidine relations.

It is noteworthy that the effect of thyroid feeding did not become evident until the second day. This was usually though not invariably observed as a few records showed a response on the first day.

For the determination of the effect of thyroid feeding on creatinuria when creatine precursors from exogenous sources were available we used pigs which did not show any increase in creatinuria after moderate casein feeding. When this fact had been established with an animal we gave it the thyroid with casein and later thyroid alone. The casein was always given 1 day after the thyroid as our previous results had shown that the thyroid effect was usually delayed 1 day.

The results of these trials are shown in Tables IV, V, and VI. In Table IV, taking the average of 3 days, there were produced 166 mg. more creatine when casein plus thyroid was fed than when thyroid was fed alone. In Table V the increase was 400 mg. daily for 2 days but in Table VI there is a balance of 60 mg. for 3 days in the opposite direction. The latter is too small to have any contradictory significance in our conclusion that thyroid medication may affect the exogenous as well as the endogenous arginine metabolism, yet it does show that the effect is not of such great magnitude that it is always manifested.

TABLE IV.

Pig, male, same individual as used in experiment shown in Table III. Energy intake as starch or its equivalent, 70 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary changes.
	cc.	*gm.*	*gm.*	*gm.*	
4	1,300	1.35	0.658	0.166	
5	1,750	1.22	0.653	0.158	105 gm. casein.
6	1,200	1.92	0.652	0.150	
7	1,250	2.00	0.660	0.150	
8	1,400	1.82	0.693	0.154	4 gm. thyroid.
9	1,400	1.96	0.714	0.184	105 gm. casein.
10	1,375	5.28	0.630	1.042*	
11	1,550	4.84	0.762	0.928	
12	1,750	2.97	0.751	0.355	
13	1,650	2.44	0.651	0.159	
14	1,650	1.98	0.732	0.200	
15	1,500	1.65	0.666	0.160	
16	1,500	1.89	0.702	0.165	4 gm. thyroid.
17	1,700	2.89	0.727	0.707	
18	1,550	2.35	0.680	0.707	
19	1,500	2.37	0.630	0.412	
20	1,500	1.50	0.588	0.169	

* Animal had diarrhea, but not severe enough to interfere with the urine analyses.

TABLE V.

Pig, male, weight 36 kilos. Energy intake as starch or its equivalent, 68 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary changes.
	cc.	*gm.*	*gm.*	*gm.*	
6	2,200	2.66	1.180	0.140	
7	2,000	2.28	1.080	0.160	
8	1,550	5.02	1.123	0.124	210 gm. casein.
9	1,950	6.35	1.014	0.132	
10	1,800	3.74	0.990	0.160	
11	2,000	2.66	1.010	0.110	2 gm. thyroid.
12	1,850	10.02	1.063	0.786	210 gm. casein.
13	2,550	8.21	1.060	0.317	
14	1,900	3.49	0.912	0.085	
15	2,150	2.66	1.130	0.097	
16	2,100	2.31	1.102	0.105	2 gm. thyroid.
17	2,500	2.12	0.900	0.140	
18	2,700	2.22	1.084	0.152	
19	2,000	2.11	1.063	0.140	

TABLE VI.

Pig, male, weight 28 kilos. Energy intake as starch or its equivalent, 68 calories per kilo.

Day of starch feeding.	Volume of urine.	Urinary nitrogen.	Preformed creatinine.	Creatine as creatinine.	Dietary changes.
	cc.	*gm*	*gm.*	*gm.*	
7	1,300	1.50	0.486	0.144	
8	1,500	1.32	0.450	0.145	
9	1,000	2.10	0.450	0.145	105 gm. casein.
10	1,300	2.60	0.437	0.134	
11	1,100	1.82	0.407	0.115	
12	1,350	2.56	0.437	0.128	4 gm. thyroid.
13	1,250	5.20	0.470	0.460	105 gm. casein.
14	1,550	4.06	0 490	0.254	
15	1,500	2.70	0.410	0.178	
16	1,550	2.32	0.428	0.123	
17	1,400	1 93	0.487	0.134	
18	1,450	2.37	0.449	0.142	
19	1,400	2.88	0.488	0.150	4 gm. thyroid.
20	1,700	3.29	0.441	0.456	
21	1,550	3.03	0.485	0.376	
22	1,200	2.49	0 393	0.243	
23	1,300	2.13	0.421	0.105	

SUMMARY.

The feeding of sheep's thyroid to a pig on a nitrogen-free diet calls forth a marked stimulation of creatine formation. This is accentuated when creatine precursors from exogenous sources are available. It is suggested that creatine formation is primarily dependent upon the balance that obtains between the arginase and oxidative systems whereby arginine is destroyed. On these premises arginine from exogenous sources is not metabolized into creatine in the same proportions as arginine from endogenous sources because this balance varies in different organs. Furthermore, it is suggested that the thyroid principle may be active in causing creatine formation by accelerating the oxidative system of arginine destruction at the expense of the effect of arginase and that in the thyroid mechanism is to be sought the variable responsible for the difference in reaction of men and women to protein feeding.

Creatinuria is looked upon as the result of the accumulation of creatine up to and beyond the threshold of its excretion. Usually this is prevented by the prevalent rate of conversion of creatine into creatinine which appears to be an invariable reaction.

BIBLIOGRAPHY.

1. Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265, and unpublished data.
2. Thompson, W. H., *J. Physiol.*, 1915, li, 111.
3. Gross, E. G., and Steenbock, H., *J. Biol. Chem.*, 1921, xlvii, 33.
4. Denis, W., *J. Biol. Chem.*, 1917, xxx, 47.
5. Magnus-Levy, A., *Z. klin. Med.*, 1897, xxxiii, 269.
6. Andersson, J. A., and Bergman, P., *Skand. Arch. Physiol.*, 1898, viii, 326.
7. Schöndorff, B., *Arch. Physiol.*, 1897, lxvii, 395.
8. Gluzinski, L. A., and Lemberger, I., *Centr. inn. Med.*, 1897, xviii, 89.
9. Voit, F., *Z. Biol.*, 1897, xxxv, 116.
10. Farrant, R., *Brit. Med. J.*, 1913, ii, 1363.
11. Underhill, F. P., and Saiki, T., *J. Biol. Chem.*, 1908–09, v, 225.
12. Cramer, W., and Krause, R. A., *Proc. Roy. Soc. London, Series B*, 1912–13, lxxxvi, 550.
13. Marshall, E. K., Jr., *J. Pharm. and Exp. Therap.*, 1920, xvi, 141.

THE DISTRIBUTION OF PHOSPHORIC ACID IN THE BLOOD OF NORMAL INFANTS.

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As part of an investigation of the factors influencing the occurrence of anemia in infants and young children it was thought desirable to make a study of the various combinations of phosphoric acid in the blood, since, as has been shown,[1] one of these compounds (the organic phosphorus) is a quantitatively important constituent of the red blood corpuscles, while another (the lecithin) also present in fairly large amounts, is commonly believed to predispose to the destruction of these cells. The work was further desirable as a contribution to the comparative physiology of these compounds which is being investigated in this laboratory for the purpose of getting information regarding their functions, and especially that of the unknown organic phosphorus compound present in large amounts in the corpuscles.

The present report consists of the results of an examination of the blood of normal infants from birth up to 2 weeks of age. For the determination 15 cc. of blood were drawn from the superior sagittal sinus, prevented from clotting by the addition of minimal amounts of oxalate or citrate, and delivered at the laboratory as soon as possible afterwards—generally in 2 to 3 hours. It was then centrifuged in graduated centrifuge tubes for 10 minutes at about 4,000 R. P. M. and the levels of total blood and corpuscles read off for the purpose of determining the ratio of corpuscles to plasma. Hemolyzed blood was rejected because of the probability that significant amounts of corpuscle phosphorus had passed into the plasma. After separation of the plasma from the corpuscles, determinations of the various phosphoric acid compounds were made in each, using the methods previously described.[2]

The results of the examination are given in Table I.

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[1] Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 49.

[2] Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.

TABLE I.

Phosphoric Acid Compounds in the Blood of Infants.

Case No	Name.	Age.	Corpuscle.	Change in weight	H_3PO_4 per 100 cc.									
					Plasma.					Corpuscles				
					Total.	Acid-soluble	In-organic	Lipoid	Or-ganic.	Total	Acid-soluble.	In-organic	Lipoid.	Organic
		days	*per cent*	*gm*	*mg.*	*mg.*	*mg.*	*mg*	*mg*	*mg*	*mg*	*mg.*	*mg.*	*mg.*
	Boys.													
1	C. K.	10		3,840 3,485	29.3	13.2		16.6		208.2	137.0	14.7	62.5	122.3
3	M. A.	26	24.0	3,595 3,635	21.7	10.0	6.6	11.5	3.4	284.0	214.0	9.4	59.5	204.5
4	N. M.	13	40.5	3,490 3,460	18.5	8.8	7.5	10.7	1.3	281.0	221.0	10.8	59.5	210.2
6	S. N.	2	45.0	3,330 3,200	23.4	13.2	7.7	10.1	5.5	260.0	178.0	13.5	74.8	154.5
9	T. S.	5	51.7	5,970 5,900						234.0	175.0	26.7	52.0	148.3
13	M. N.	7	43.2	3,270 3,180	35.0	23.6	9.6	18.2	14.0	275.7	216.0		58.0	
14	M. N.	6	41.9	3,080 3,010	34.0	18.0	10.2	15.9	7.8	240.3	190.0		47.6	

15	M. A.	6	53.1	2,400 2,460	31.5	18.0	9.1	13.8	8.9	250.0	175.0		49.0	
17	P. Y.	11		3,000 3,050	30.0		10.7	17.2		240.0	195.3		46.8	
18	H. Y.	12	49.0	3,430 3,345	35.9	14.5	10.7	21.4	3.8	260.4	204.0	11.9	56.6	182.1
Average			43.5		28.8	14.9	9.0	15.0	6.4	253.4	190.5	14.5	56.6	170.3
	Girls.													
2	J. N.	12	40.5	3,370 3,370	22.5	12.8		11.1		347.0*	135.0	13.5	73.0	121.5
5	V. R.	15	40.5	3,450 3,330	23.9	13.6	8.0	10.4	5.6	221.0	151.0	14.2	60.0	136.8
8	S. Y.	10	51.3	2,810 2,720						253.0	200.0	21.4	54.3	178.6
10	R. D.	15	51.0	3,360 3,180	45.0*	25.0	13.9	17.3	11.1	240.0	187.0	10.4	53.2	176.6
12	F. S.	5	43.0	4,690 4,820	35.0	16.3	10.0	20.8	6.3	279.0	219.7		58.1	
16	D. E.	13	52.0	3,470 3,340	35.0		12.5	13.0		234.0	163.0		35.7	

Not included in average

TABLE 1—*Concluded.*

Case No.	Name.	Age.	Corpuscle.	Change in weight.	H_3PO_4 per 100 cc.									
					Plasma.					Corpuscles.				
					Total.	Acid-soluble	In-organic	Lipoid	Or-ganic.	Total.	Acid-soluble.	In-organic.	Lipoid.	Organic.
		days	*per cent*	*gm.*	*mg.*	*mg*	*mg.*	*mg.*	*mg.*	*mg*	*mg*	*mg.*	*mg.*	*mg.*
19	L. Z.	11	36.4	2,670 2,905	29.4	17.5	12.9	16.6	4.8	208.0	151.2	12.8	44.1	138.4
20	L. L.		37.2	2,670 2,765	43.1	25.0	11.6	17.5	11.4	234.3	173.6	15.1	50.0	158.5
21	W. N.	17	45.2	4,120 4,065	21.5	15.7	10.9	12.9	4.6	186.5	142.0	12.5	42.7	129.5
22	O. N.	2	45.0	2,620 2,605	24.0	17.5	11.3	10.6	5.3	198.4	143.0	13.8	55.5	130.2
23	O. N.	3	54.0	3,815 3,845	23.4	12.2	10.0	11.6	2.2	260.0	175.5	17.0	45.5	158.5
Average			45.1		30.3	17.4	11.2	14.1	6.4	231.4	167.3	14.9	52.0	147.6

DISCUSSION.

Influence of Sex.—Very little difference can be noted in the values as characteristic of the sexes. In the plasma the values for inorganic phosphorus are markedly lower in the boys than in the girls while the lipoid phosphorus is slightly higher but not significantly so. The values for organic phosphorus are the same in both and show the same wide variations. In the corpuscles the only striking difference is in the organic phosphorus which is considerably higher in the boys. Since, however, relatively few determinations are available much emphasis cannot be laid on these differences.

TABLE II.

Averages and Variations in the Phosphoric Acid Compounds in the Blood of Infants and of Adults.

		Plasma.					Corpuscles				
		Total.	Acid-soluble	Inorganic	Lipoid.	Organic.	Total.	Acid-soluble	Inorganic	Lipoid	Organic.
Low	Adult.....	27.6	8.4	7.0	17.5	0.2	200.0	150.0	10.9	45.0	128.0
	Infant....	18.5	8.8	3.7	10.1	1.3	186.5	135.0	9.4	42.7	121.5
Average	Adult.....	34 0	11.4	9.9	23.5	1.5	248.0	187 0	17.2	57.0	169.5
	Infant....	29.7	16.5	9.6	14.8	7.0	241.4	179.6	14.3	56.0	156 0
High	Adult.....	42.2	14	14	26.2	4.0	295.0	228.0	26.8	64.0	213.0
	Infant....	43.0	25	13.9	21.4	11.4	284.0	221.0	26.7	74.8	204.5

Influence of Weight Changes.—Most of the infants were losing weight at the time the samples were taken so that little can be said with regard to the effect of gaining or losing weight on the blood phosphates. As far as can be seen, however, in infants gaining weight the values are higher than the average in the plasma and somewhat lower in the corpuscles, while in those losing weight the reverse is the case although the difference is less noticeable.

Comparison with Adults.—For convenience in comparison there are given in Table II the averages and variations in the phosphoric acid compounds of infants and adults.

Corpuscles.—The average values for total and lipoid phosphorus in the corpuscles of adult and infant agree very closely while the average inorganic value is considerably, and the organic somewhat, lower in the infant. The low values are lower in the infant than in the adult as is also the case with the high values with the exception of the lipoid phosphorus which reaches higher levels than are normally found in the adult. The latter may be due to the almost continuous absorption of fat in infants of this age, since high values for fat in blood have been found to bring about increases of lipoid phosphorus in the corpuscles.

The corpuscle values as a whole are remarkably similar in the infant and the adult and indicate that there is little if any change in the composition of the red cells after birth—a finding which is in marked contrast to that in cattle in which Meigs[3] found much higher values (nearly twice in some cases) for total phosphorus in the corpuscles of young calves than in those of cows.

Plasma.—The organic phosphorus is regularly much higher in the infant than in the adult, resulting also in a higher acid-soluble fraction. Lipoid phosphorus is much lower throughout in the infant than in the adult, in this respect agreeing with the findings of Meigs[3] with cattle in which the lipoid phosphorus of the plasma of calves is found very low, increasing up to the age of about 1 year.

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[3] Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.

THE DETERMINATION OF INORGANIC SULFATE, TOTAL SULFATE, AND TOTAL SULFUR IN URINE BY THE BENZIDINE METHOD.

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As far as its use for the analysis of urine is concerned, the benzidine method for the determination of sulfur is subject to two sources of error that may under certain conditions be serious. One of these, the contamination of the benzidine sulfate precipitate by phosphate, has been recognized before, and can be eliminated by adding acid before precipitating with benzidine. For this purpose Rosenheim and Drummond[1] recommend the addition of hydrochloric acid to the urine until Congo red paper shows an acid reaction. Gauvin and Skarzynski,[2] on the other hand, add the same quantity of hydrochloric acid in every instance, without the use of an indicator, and this scheme has also been adopted by Drummond[3] in a more recent modification of the Rosenheim and Drummond method on a smaller scale. In the methods for inorganic sulfate proposed by all these writers, the concentration of benzidine during the precipitation is about the same (0.017 to 0.02 N), but the concentration of hydrochloric acid is much less uniform. In Table I are collected data showing the concentrations of sulfuric acid, benzidine, and hydrochloric acid existing during the precipitation from sulfate solutions by these three methods. The figure for hydrochloric acid given in the table for Rosenheim and Drummond's method holds also with urine, since in this method the urine is acidified before adding the benzidine reagent. In the other two methods, inasmuch as this step is omitted, the concentration of hydrochloric acid during the precipitation of benzidine sulfate from urine is less than stated by an amount that varies with the character of the urine.

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[1] Rosenheim, O., and Drummond, J. C., *Biochem. J.*, 1914-15, viii, 143.

[2] Gauvin, R., and Skarzynski, V., *Bull. Soc. chim.*, 1913, 4th series, xiii, 1121.

[3] Drummond, J. C., *Biochem. J.*, 1915, ix, 492.

In spite of these differences, all three methods have given results that agree fairly well with the gravimetric method, but they have done so only because the comparisons have been confined to urines that are very much alike with respect to the factors that affect the accuracy of the determination. As long as their use is restricted to ordinary 24 hour urines, any one of a great many possible modifications would suffice to avoid trouble due to the presence of phosphate, simply because in such urines there is never a very large amount of phosphate in proportion to the sulfate content. But the much more exacting requirements of the less uniform urines obtained in short period metabolism experiments are satisfactorily met neither by any modification of the benzidine method that has been suggested so far, nor by any that I have been able to find that does not involve the preliminary removal of the phosphate.

TABLE I.

Method.	Sulfuric acid.	Benzidine.	Hydrochloric acid
	N	N	N
Rosenheim and Drummond.............	0.004–0.007	0.017	0.024
Drummond............................	0.003–0.010	0.017	0.084
Gauvin and Skarzynski................	0.002–0.003	0.020	0.031

If phosphate were the only source of trouble, it would be easy to devise a method that would do for all circumstances that are likely to arise, although such a method would call for a more precise adjustment of the acidity than has heretofore been thought necessary. But unfortunately there is a second cause of error, in some respects even more troublesome. This is the increase in solubility of benzidine sulfate due to the presence of chloride, which often exists in urine in sufficient concentration to be injurious in this way. That this may rarely or never be the case in 24 hour urines presumably accounts for the fact that the effect of chloride has previously escaped notice in connection with urine analysis.

In 1 hour urines, on the other hand, for each mg. of sulfur in the form of inorganic sulfate, there may be as much as 5 mg. of inorganic phosphorus and 30 mg. of chlorine, and even more under conditions that can hardly be called unusual, although they may not be especially frequent. The influence of the addition of sodium chloride and disodium phosphate on the analysis of sulfate solutions by Drummond's method will be seen from the figures recorded in Table II. That the same factors are not without considerable effect on sulfate determinations in urine is evident from the results in Table III, all obtained with one sample of urine, which was analyzed alone (Experiments 1 and 4) and after the addition of disodium phosphate (Experiments 2 and 5) and of sodium chloride (Experiment 3). Depending upon the amount of phosphate and chloride present (and the conditions in this respect are not extreme for 1 hour periods) the figures vary by more than 10 per cent.

TABLE II.

Analysis of Sodium Sulfate Solution by Drummond's Method.

Composition of solution.			Sulfur found.
Sulfur.	Phosphorus.	Chlorine.	
mg.	*mg.*	*mg.*	*mg.*
1	0	0	1.00
1	5	0	1.04
1	0	30	0.97
1	0	60	0.91

TABLE III.

Analysis of Urine by Drummond's Method.

Experiment No.	Urine.	Inorganic phosphorus	Chlorine.	Inorganic sulfate sulfur found.	
	cc.	*mg.*	*mg.*	*mg.*	*mg per 100 cc.*
1	1	0.66	14.8	0.674	67.4
2	1	3.36	14.8	0.678	67.8
3	1	0.66	34.8	0.642	64.2
4	2	1.32	29.6	1.39	69.5
5	2	6.72	29.6	1.44	72.0

A considerable experience with short period metabolism experiments has led me to the conclusion that any sulfur method, to be safe for such work, must give accurate results in the presence of 10 mg. of phosphorus or 60 mg. of chlorine for each mg. of sulfur in the form of inorganic sulfate. Many different benzidine reagents have been tried, under all sorts of conditions, but none has been found equal to these requirements, and it is apparently necessary to accept the fact that both these difficulties cannot be successfully contended with at the same time. Once the phosphate has been removed, the situation is much simplified, as it is then possible to avoid trouble from the presence of chloride by precipitating the benzidine sulfate at a much lower acidity than would otherwise be permissible.

Removal of Phosphate.

For the removal of phosphate from urine as a preliminary to the precipitation of sulfate with benzidine, nothing has been found equal in effectiveness to magnesia mixture in some form, and it is fortunately possible to precipitate the phosphate nearly quantitatively as magnesium ammonium phosphate without the introduction of injurious quantities of electrolytes, which, like sodium chloride, would prevent the complete precipitation of benzidine sulfate. This is accomplished by shaking the urine (previously neutralized with ammonia) with basic magnesium carbonate in the presence of a small amount of ammonium chloride. The whole process of precipitation and filtration requires only a very few minutes. If the first 15 or 20 cc. of filtrate are poured back on the paper and filtered again, the solution will percolate through a layer of magnesium carbonate mixed with triple phosphate crystals, and this is a particularly effective way of removing phosphate. The final filtrate should then contain less then 0.1 mg. of inorganic phosphorus in 5 cc.

The urine must be fairly dilute before the phosphate is removed, for magnesium ammonium phosphate crystallizes with 8 molecules of water, and the removal in this way of more than 0.2 per cent of phosphorus would appreciably alter the concentration of sulfate in the filtrate.

In the following directions for preparing the essentially phosphate-free filtrate, the quantities prescribed are sufficient for duplicate determinations of all three forms of sulfur (inorganic sulfate, total sulfate, and total sulfur).

Transfer to a 50 cc. volumetric flask sufficient urine to contain between 5 and 10 mg. of sulfur in the form of inorganic sulfate, and dilute to about 25 cc. with water. Add 1 drop of phenolphthalein solution and 1 drop of concentrated ammonium hydroxide (or as much as is necessary to make the solution faintly pink), followed by 5 cc. of a 5 per cent solution of ammonium chloride. Make up to the mark, mix, and pour the solution into a dry Erlenmeyer flask containing about 0.65 gm. of *finely powdered* basic magnesium carbonate.[4] Shake for 1 minute, and transfer to a 9 cm. filter paper enough of the suspension to fill the paper nearly to the top. Allow this first filtrate to drain back into the Erlenmeyer flask, and then filter the entire suspension through the same paper into a dry container.

In case the urine is already extremely dilute, the phosphate can be precipitated without appreciably altering the concentration by using solid ammonium chloride (0.25 gm.) instead of a solution. Urines obtained in short period experiments are sometimes so dilute as to make this modification necessary.

The filtrate, prepared as described above, is now used for all three sulfur determinations.

Determination of Sulfur in the Phosphate-Free Filtrate.

Inorganic Sulfate.—Pipette 5 cc. of the filtrate into a 100 cc. beaker. Add 2 drops of a 0.04 per cent alcoholic solution of brom-phenol blue[5] and 5 cc. of water. Then add approximately N HCl, drop by drop, until the solution is yellow without a trace of blue. Run in, from a pipette, 2 cc. of benzidine reagent,[6] and let stand for 2 minutes. Finally, add 4 cc. of 95 per cent acetone, and let stand for 10 minutes more. Filter through a mat of paper pulp in a special filtration tube (described below). Wash the beaker and the filter, first with three 1 cc. portions of 95 per cent acetone, and then once with 5 cc. Transfer about 2 cc. of water to the filtration tube, and poke the precipitate and mat through the hole in the lower end into a large Pyrex test-tube (200 by 20 mm.), using a sharpened nichrome wire. Rinse off the wire with a few drops of water, and heat the contents of the test-tube just to boiling, leaving the filtration tube suspended in the mouth of the test-tube. Add 2 drops of a 0.05 per cent aqueous solution of phenol red (monosodium salt),[5] and run in from a micro-burette,[7, 8] through the filtration tube, about 1 cc. of 0.02 N NaOH. Rinse down the wall of the filtration tube with 2 or 3 cc. of water from a wash bottle, heat again to boiling until steam escapes actively from the test-tube, and rinse a second time with sufficient water to bring the total volume up to about 10 cc. This treatment should suffice to remove all traces of precipitate from the filtration tube, which may now be removed, and the titration with 0.02 N NaOH continued. When the color begins to change from yellow to red, again heat to boiling, and pour the hot solution into the beaker (in which the precipitation took place) and back.[9] This will decompose any trace of precipitate that may have adhered to the wall of the beaker. From this point on the standard alkali should be added, not more than 0.02 cc. at a time, until the solution acquires a definite pink color, which further boiling does not discharge.

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[4] This reagent must obviously be free from sulfate. Baker's analyzed magnesium carbonate has proved satisfactory.

[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 63.

[6] Suspend 4 gm. of benzidine in about 150 cc. of water in a 250 cc. volumetric flask. Add 50 cc. of N HCl (standardized). Shake until dissolved, and make up to volume. Filter if necessary.

Total Sulfate.—To 5 cc. of the filtrate in a 100 cc. beaker add 1 cc. of 3 N HCl (approximate). Heat on the water bath until the solution has evaporated to dryness, and for 10 minutes longer. Immediately add 10 cc. of water, and break up the residue by rotating the beaker. Add 2 cc. of the benzidine reagent and (2 minutes later) 4 cc. of acetone, exactly as in the method for inorganic sulfate, and complete the determination as described above.

Total Sulfur.—Transfer 0.25 cc. of Benedict's total sulfur reagent[10] to a 6 cm. evaporating dish, and add 5 cc. of the urine

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[7] Folin, O., and Peck, E. C., *J. Biol. Chem.*, 1919, xxxviii, 289.

[8] Fiske, C. H., *J. Biol. Chem.*, 1921, xlvi, 285.

[9] This step may be avoided by conducting both precipitation and titration in a large lipped test-tube, but except for the inorganic sulfate determination a beaker is on the whole more convenient.

[10] Benedict, S. R., *J. Biol. Chem.*, 1909, vi, 363. The reagent contains 20 gm. of copper nitrate crystals and 5 gm. of potassium chlorate per 100 cc. A blank (gravimetric) must, of course, be run on the reagent unless the copper nitrate is free from sulfate.

filtrate. Evaporate to dryness, preferably on an electric hot plate at low heat. When the mixture has become dry, increase the heat by steps to the maximum, and finish the ignition with a microburner, allowing 2 minutes at red heat after the contents of the dish have become thoroughly black. Cool for 5 minutes. Add 1 cc. of 3 N HCl, and evaporate to dryness on the hot plate (low heat). When the residue is thoroughly dry, dissolve and wash into a 100 cc. beaker with five 2 cc. portions of water. Add 1 drop of N HCl, and precipitate with the benzidine reagent and acetone as in the other two methods. The rest of the determination is likewise the same as before, with the single exception that 2 cc. of 50 per cent acetone should be used in place of the first of the three 1 cc. portions of 95 per cent acetone, otherwise it will be impossible to wash the filter free from copper.

The amount of sulfur in the 5 cc. of filtrate analyzed is in each case obtained (in mg.) by multiplying the titration figure by 0.32.

DISCUSSION.

Use of Acetone.—At a time when there was still some hope of avoiding the necessity of removing the phosphate, the addition of acetone during the precipitation with benzidine was introduced for the purpose of diminishing the solubility of the precipitate. Although this may not be altogether necessary under the conditions finally adopted, the modification has been retained, and the same liquid used for washing the precipitate, for various reasons. Washing with a saturated solution of the precipitate is a thing to be avoided whenever possible, and acetone is to be preferred on that account. Since acetone wets glass more readily than does water, it does not collect in drops on the wall of the beaker, a matter of some consequence when a fairly large surface must be washed with a small volume of liquid. But the most important consideration of all is that the use of acetone altogether prevents the benzidine sulfate from assuming the form of large flakes, which can be decomposed only by prolonged boiling at the end of the titration, and on account of which Rosenheim and Drummond,[1] who wash the precipitate with a saturated solution of benzidine sulfate, have been obliged to recommend that the filter never be allowed to be sucked dry.

Indicator.—Boiling aqueous solutions of purified benzidine, at concentrations corresponding with the conditions at the end-point of the titration described above, give a brownish color with phenol red, intermediate between yellow and red. The addition of less than 1 per cent of one equivalent of sodium hydroxide is sufficient to change the color to a definite pink, whereas phenolphthalein under these conditions is still colorless. Since phenol red is besides a much more brilliant indicator, it is to be preferred on all counts.

Filtration Tube.—This is, in principle, the same as the tube recently described in connection with a method for the determination of inorganic phosphate in urine.[8] The narrow tube recommended for the filtration of magnesium ammonium phosphate is readily clogged by the much more compact benzidine sulfate precipitate, and for sulfate determinations it should be replaced by one considerably larger in diameter. The tubes that have been found most convenient are made from glass tubing[11] 15 mm. in internal diameter, shrunken at one end so as to leave a hole 3 mm. in diameter, cut to a length of 70 mm., and flanged at the cut end. The somewhat elongated tip shown in Fig. 1 gives the best results.

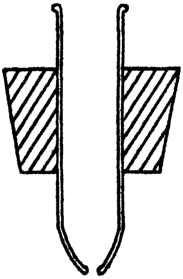

FIG. 1. Filtration tube (one-half natural size).

In using this tube, only enough paper pulp should be introduced to form a thin cup-shaped mat lining the constricted tip. It is neither necessary nor desirable to fill the tip with pulp. The mat having been prepared, the tube should be filled with the solution to be filtered *before* starting the suction [illegible] the suction, when it is started, should be very gentle. Any attempt to hasten the filtration by applying strong suction is almost certain to produce the opposite result by packing down the precipitate.

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[11] Pyrex tubing is preferable. Since the tube is subjected to the action of steam during the titration, soft glass should not be used.

Adjustment of Acidity.—The purpose of the preliminary acidification (to brom-phenol blue) in the determination of inorganic sulfate is to neutralize any basic substances that may be present and to liberate weak acids from their salts. No appreciable amount of the hydrochloric acid in the benzidine reagent will then be neutralized by constituents of the urine filtrate. In the method for total sulfate, this adjustment is made automatically by evaporating the urine with hydrochloric acid.

TABLE IV.

Urine No		Inorganic sulfate S.	Inorganic P / Inorganic sulfate S	Chlorine / Inorganic sulfate S	Sulfur per hr.					
					Inorganic sulfate.		Total sulfate.		Total sulfur.	
					Gravimetric.	Titration.	Gravimetric.	Titration.	Gravimetric.	Titration.
		mg per 100 cc			*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	(*a*)	21.0	1.8	14	15.0	15.4	17.4	17.3	21.1	21.1
	(*b*)		10.0	14		15.4		17.3		20.9
	(*c*)		1.8	60		15.3		17.3		21.1
2	(*a*)	9.5	0.9	13	22.7	22.6	25.4	25.6	32.6	32.5
	(*b*)		10.0	13		22.8		25.6		
	(*c*)		0.9	60		22.4		25.3		32.3
3	(*a*)	8.5	1.0	18	14.3	14.8	17.7	17.8	22.1	22.4
	(*b*)		10.0	18		14.7		17.9.		22.3
	(*c*)		1.0	60		14.7		18.0		22.3
4	(*a*)	20.5	0.8	9	34.5	34.7	37.5	37.7	42.0	41.7
	(*b*)		10.0	9		34.8		37.7		41.8
	(*c*)		0.8	60		34.3		37.7		42.2

Hydrolysis of Ethereal Sulfate.—Evaporation with hydrochloric acid is substituted for the usual boiling mainly because the additional acid required for hydrolysis would be enough to cause low results in the presence of large amounts of chloride.

[illegible]*thod.*—The only special modification introduced in this determination is the removal of the excess hydrochloric acid, after dissolving the residue of copper oxide, by evaporation to dryness instead of by neutralization with alkali. Neutralization would introduce more sodium chloride, which is undesirable for reasons that have been mentioned. After the excess acid has been removed by evaporation, the residue is at times so nearly neutral that a drop of dilute acid should be added (as stated) before running in the benzidine reagent.

Results.

The method as described gives satisfactory results in the presence of 10 mg. of inorganic phosphorus or 60 mg. of chlorine for each mg. of sulfur in the form of inorganic sulfate. A series of analyses is given in Table IV. Each of the urines was analyzed first in the manner described above (*a*), secondly after adding sufficient disodium phosphate to bring the inorganic phosphate content up to the stated figure (*b*), and finally after the addition of enough sodium chloride to make the ratio of chlorine to inorganic sulfate S equal to 60 (*c*). For comparison the same urines were analyzed gravimetrically (Folin's[12] method for inorganic and total sulfate; Benedict's[10] method for total sulfur).

Differences greater than about 1 per cent sometimes occur in the determination of inorganic sulfate, especially when the sulfur excretion is small, however closely duplicates by each method may agree. In such cases there is apparently no way of deciding which method is the more accurate. Discrepancies of this nature have not been observed in the determination of total sulfate or total sulfur.

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[12] Folin, O., *J. Biol. Chem.*, 1905, i, 131.

BASAL METABOLISM OF NORMAL WOMEN.*

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The primary purpose of this investigation was to find out whether there is any regular periodic variation in the basal metabolism of normal women during menstruation. The figures obtained show the lack of such periodicity. They bring out also the marked day by day variation in basal metabolism which may be expected at any time, and the 216 observations add to the already accumulated data on standards for women. These three points will be taken up successively.

Procedure for Determining Metabolism.

Method.

The Benedict portable respiration apparatus was used to determine the basal metabolism. In general, the procedure of the Nutrition Laboratory for determining oxygen consumption was followed (1, 2). The subject, in a postadsorptive condition, took a 30 minute rest period in a quiet room preceding the two 10 minute observations. The initial reading for the volume of oxygen was taken after the subject was connected with the air current at the end of a series of regular expirations, and at the same instant a stop-watch was started. With a second stop-watch another reading was taken so that duplicate records were made for each 10 minute period. The day's basal metabolism was taken as the average of the duplicates of the two 10 minute periods. If for any subject checks were not obtained on the first two periods, a third period was observed. Very occasionally a whole day had to be discarded because of failure to obtain satisfactory duplicates. The results are given only when duplicate readings agree within 5 per cent. No attempt was made to determine the carbon dioxide. Benedict (1) has found that the error, made in assuming a constant respiratory quotient, when the subject has been 12 or more hours without food, is slight. We have assumed the customary respiratory quotient of 0.82 and the factor 4.825 calories per liter of oxygen.

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* The work reported in this paper will form part of the thesis to be submitted by Marie Dye for the degree of Doctor of Philosophy, University of Chicago.

Subjects.—Our seventeen subjects were faculty members or students at the University of Chicago from 21 to 44 years old. All were in normal physical condition, and continued their usual academic work during the menses, four of them stating that they felt absolutely no discomfort or disturbance of any kind (Nos. 12, 13, 14, and 15) and the others experiencing more or less fatigue or lassitude. From one to three complete periods were studied with pre- and intermenstrual observations on each of fourteen of the subjects. One case of amenorrhea (No.1) was observed for 26 almost successive days. Two intermenstrual studies of 10 days each were made (Nos. 3 and 8).

Experimental Data.

The detailed results of all observations are given in Table VI, listing the subjects according to age. The age is that at the nearest birthday, the weight is taken nude, and the surface area is read from the DuBois height-weight chart (3).

Comparison of Metabolism during Menstrual and Intermenstrual Phases.

Averages for Menstrual and Intermenstrual Phases.—In Table I are brought together the averages of all the observations made on the fourteen women during the menstrual and the intermenstrual periods. The figures show no consistent variation. In only six of the subjects is the difference between averages for the two phases greater than 3 per cent, and of these six, some have the higher metabolism during menstruation and some in the intermediate period. The average of all the subjects gives a 1.6 per cent lower metabolism during menstruation than at other times and practically the same decrease when only the first 2 menstrual days are included. This difference is too small to be considered significant.

Periodicity of Metabolism during Premenstrual and Menstrual Phases.—There also seems to be no regular, rhythmic, day by day variation. The curve of the daily observations is far from smooth. Table II, which lists the daily metabolism immediately before and during menstruation, gives a confused sense of irregularity rather than of periodicity. With a number of subjects a high metabolism day can be found just before or at the beginning of menstruation, but so also can a low metabolism day. Moreover, the high day connected with menstruation is only sometimes higher than other days observed. The only possible conclusion seems to be that the variation connected with the menstrual cycle is irregular and no greater than at other times.

TABLE I.

Comparison of Average Heat Production during Menstrual and Intermenstrual Phases.

Subject.	Age.	Height.	Weight.	Surface area.	Intermenstrual.		Menstrual.			First 2 menstrual days.		
					Days observed.	Calories per 24 hrs.	Days observed.	Calories per 24 hrs.	Above intermenstrual.	Days observed.	Calories per 24 hrs.	Above intermenstrual.
		cm.	*kg.*	*sq. m.*	*No*		*No.*		*per cent*	*No.*		*per cent*
2	24	152	66.7	1.63	3	1,407	6	1,445	2.7	2	1,430	1.6
4	25	164	64.1	1.70	2	1,277	3	1,332	4.3	2	1,357	6.3
5	26	161	50.1	1.51	5	1,281	11	1,231	−3.9	6	1,229	−4.0
6	28	148	53.0	1.44	4	1,137	8	1,132	−0.4	3	1,170	2.9
7	28	160	61.0	1.63	3	1,353	6	1,318	−2.6	4	1,322	−2.3
9	29	149	51.0	1.43	3	1,238	6	1,272	2.7	4	1,281	3.5
10	30	170	61.7	1.70	2	1,537	7	1,468	−4.5	3	1,505	−2.1
11	32	151	43.0	1.34	3	1,135	3	1,068	−5.9	2	1,052	−7.3
12	33	168	62.0	1.69	2	1,360	3	1,337	−1.7	2	1,315	−3.3
13	33	173	79.0	1.93	3	1,633	8	1,551	−5.0	2	1,562	−4.3
14	37	162	75.5	1.80	2	1,500	5	1,454	−3.1	4	1,454	−3.1
15	40	166	66.6	1.74	2	1,510	3	1,437	−4.8	2	1,442	−4.5
16	41	158	70.0	1.71	2	1,347	4	1,329	−1.3	2	1,310	−2.7
17	44	162	49.1	1.49	7	1,203	11	1,224	1.7	5	1,210	0.5
Average.						1,351		1,328	−1.6		1,331	−1.3

We have been able to find in the literature only three other series of observations on basal metabolism during menstruation, a long series by Zuntz (4) in 1906, two observations by Gephart and Du Bois (5) in 1916, and a preliminary report by Ford in a recent paper by Snell, Ford, and Rowntree (6). Zuntz determined the carbon dioxide output and part of the time the oxygen consumption for two women for 97 days almost without a break, including three menstrual periods with inter-, pre-, and

TABLE II.

Daily Variations during Premenstrual amd Menstrual Phases in Calories per 24 Hours.

Subject.	2 days before menstruation.	Day before menstruation.	Day of menstruation.					
			1st	2nd	3rd	4th	5th	6th
2	1,390*	1,355	1,500	1,360	1,440	1,440	1,440	1,490
4	1,375*		1,355	1,360	1,280			
5 (I)	1,375*	1,375	1,300	1,390	1,330	1,230		
5 (II)		1,370	1,235	1,195	1,195	1,330		
5 (III)	1,100	1,135	1,110	1,145	1,080			
6 (I)			1,200		1,090	1,070	1,125	
6 (II)	1,200	1,145	1,210	1,100	1,140	1,125		
7 (I)			1,390	1,310	1,310			
7 (II)	1,320	1,320	1,300	1,290	1,310			
9 (I)		1,250	1,310	1,260	1,270			
9 (II)		1,230	1,265	1,290	1,235			
10 (I)	1,540	1,515	1,490	1,425	1,465	1,475		
10 (II)	1,585	1,425	1,600		1,420	1,400		
11	1,125	1,105	1,085	1,020	1,105			
12			1,320	1,310	1,380			
13 (I)		1,650	1,620	1,520	1,620	1,515		
13 (II)		1,585	1,575	1,535	1,535	1,490		
14 (I)		1,390	1,455	1,390	1,455			
14 (II)		1,460	1,480	1,490				
15	1,410		1,425	1,460	1,425			
16	1,290	1,360	1,370	1,250	1,320	1,375		
17 (I)		1,145	1,315		1,270	1,300		
17 (II)		1,080	1,140	1,140		1,220	1,235	
17 (III)	1,260	1,190	1,235	1,220	1,220	1,175		

* The actual observations of cc. of oxygen per minute from which these figures were calculated were for No. 1: 202, 202, 200, and 197; for No. 2: 193, 198, 200, and 200; and for No. 3: 197, 198, 200, and 199. These may be taken as typical of the range in our duplicate observations.

postmenstrual observations. His conclusions, and also those of Gephart and Du Bois, are in line with ours—that there is no regularity of variation.

Snell, Ford, and Rowntree, on the other hand, conclude that menstruation "does affect the basal metabolic rate of women at times in health and disease." Ford concluded for eight cases out of ten studied that a rather constant rise occurs during menstruation or in the premenstrual period, the rise being followed by a postmenstrual fall. Two of the ten cases showed a drop rather than a rise. The details of this work have not yet been published. We have no information of our subjects in regard to postmenstrual metabolism, but we do not find regularly the menstrual or premenstrual rise that Ford does.

Daily Variation in Metabolism.

As already pointed out in the discussion of menstrual variation, the extremes of metabolism of these subjects on different days show a wide range. Since this is as marked during the intermenstrual as the premenstrual and menstrual phases, no distinctions of phases are made in this part of the discussion. The maximum and the minimum metabolism observed at any time for each subject, including the three subjects observed without menstrual phase, are brought together in Table III. The percentage of the maximum above the minimum is very high in many cases. The highest difference is 28.8 per cent, the lowest 7.4 per cent, and the average 13.2 per cent. Only six of the seventeen subjects show a range lower than 10 per cent which the clinician often uses as his criterion of normality as compared with a standard. Even if the variations are calculated from the average for the individual, instead of by comparison of maximum and minimum values, three of the subjects still have a greater range than 10 per cent.[1] Our observations, therefore, emphasize the importance of taking several days' rather than a single day's observation to arrive at what is average for an individual.[2]

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[1] Compare the recommendation of Boothby (7) in a recent article: "all patients having a rate ranging between +10 and +20 per cent. should have the test repeated on a subsequent day."

These observations on the high variation which may be expected for an individual on different days merely confirm those given by Benedict in 1915 (8). At that time he listed the extreme variations for a large number of his subjects who had been studied 5 days or more. His greatest variation above this minimum was 31.3 per cent, his lowest 3.5 per cent, and his average 13.9 per cent.

TABLE III.

Extreme Variations.

Subject.	Total No. of days observed	Heat production per day.			Variation of maximum above minimum.		Extreme variation from average.	
		Maximum	Minimum.	Average.				
		calories	*calories*	*calories*	*calories*	*per cent*	*calories*	*per cent*
1	26	1,320	1,125	1,239	195	17.3	−114	−9.2
2	14	1,500	1,355	1,413	145	10.7	87	6.2
3	10	1,345	1,125	1,205	220	19.6	140	11.6
4	6	1,375	1,260	1,321	115	9.1	−61	−4.6
5	25	1,390	1,080	1,254	310	28.8	−174	−14.0
6	14	1,210	1,070	1,140	140	13.1	−70	−6.1
7	13	1,390	1,290	1,335	100	7.8	55	4.1
8	10	1,495	1,340	1,396	155	11.6	99	7.1
9	11	1,310	1,220	1,256	90	7.4	54	4.3
10	13	1,600	1,400	1,493	200	14.2	107	7.2
11	8	1,170	1,020	1,105	150	14.7	−85	−7.7
12	5	1,430	1,290	1,346	140	10.8	84	6.2
13	13	1,660	1,490	1,580	170	11.4	−90	−5.7
14	9	1,510	1,390	1,457	120	8.6	−67	−4.6
15	7	1,520	1,410	1,457	110	7.8	63	4.3
16	9	1,375	1,250	1,331	115	9.5	−81	−6.1
17	29	1,315	1,080	1,218	235	21.8	−138	−11.3
Average..						13.2		

We have no explanation to offer for the wide variation, with the possible exception of the subject showing the widest range—

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[2] If the 1 exceptionally high day for Subject 3 is omitted (discussed below in connection with pulse rate), her average becomes 1,189 calories per 24 hours, her variation of the maximum above the minimum 125 calories, or 11.1 per cent, and her greatest variation from her average—64 calories, or 5.4 per cent.

Subject 5 with 28.8 per cent. Her average metabolism in winter (February and March) was 1,302, distinctly higher than in the summer (July) 1,145. Yet Subject 10, the only other observed both winter (early March) and summer (June), averaged the same at the two seasons (1,486 and 1,487). Another possible though not completely satisfactory reason for the differences in Subject 5 was that she was observed very early in the morning, 6.30 or 7.00, at the time when her metabolism was running lowest, and sometimes later in the day, about 11 a. m., when it ran high. She had been fasting, of course, since the night before, and had the usual rest period, but at the later date she had been attending classes and doing laboratory work before coming for the measurement of her metabolism. The late figures, however, are by no means always higher. With only a very few exceptions, all observations on other subjects were made early in the morning, usually from 7.00 to 8.30.

The diet of the subjects was not controlled, but it is not thought that there were any marked changes in it during the experiments. Neither were there marked changes of occupation nor of general health.

We wish to call special attention to the series of observations on Subject 1 (see Table VI), a young Chinese woman of excellent mentality and apparently good general health in spite of a tendency to amenorrhea. Her basal metabolism was observed daily for 26 almost consecutive days, without menstrual period. As far as we know, this is the longest series made on a woman, with the exception of Zuntz's observations. She showed much day by day variation with sudden rises and falls. There are only three subjects of the total seventeen who show a wider range than hers.

The wide range is also shown in two other women for whom we have a fairly long series of consecutive observations, Nos. 3 and 8. For No. 8 with 10 consecutive days, the variation is 11.6 per cent, and for No. 3 with 7 consecutive days, 11.1 per cent, or 19.6 per cent if the high observation of the previous month is included.

Comparison with Normal Standards for Metabolism.

The two commonly used series of "normal" standards for the basal metabolism of women are Benedict's and Du Bois'. Benedict's standards, published as "multiple prediction tables"(9) are based on observations of 103 women made in the Nutrition Laboratory. They give the basal metabolism in calories per 24 hours for different ages, heights, and weights. Du Bois' (10) standards are expressed in calories per square meter per hour, and are computed from those for men on the assumption that the basal metabolism of women is 7 per cent lower than that of men. As the height and weight variables are included in the surface area these standards of Du Bois vary only with age, ranging for our group from 37.0 calories for 20 to 30 years to 36.0 calories for 40 to 50 years.

TABLE IV.

Comparison of Observed Metabolism with that Predicted by Benedict and by Du Bois.

Subject.	Heat production per day.				Heat production per sq. m. per hr.*			
	All observations	Benedict's prediction.	Actual less calculated.	Difference.	All observations.	Du Bois' prediction.	Actual less calculated.	Difference.
	calories	*calories*	*calories*	*per cent*	*calories*	*calories*	*calories*	*per cent*
1	1,239	1,412	−173	−12.2	31.9	37.0	−5.1	−13.8
2	1,413	1,466	−53	−3.6	36.2	37.0	−0.8	−2.2
3	1,205	1,305	−100	−7.6	34.2	37.0	−2.8	−7.6
4	1,321	1,459	−138	−9.5	32.3	37.0	−4.7	−12.7
5	1,254	1,311	−57	−4.3	34.7	37.0	−2.3	−6.2
6	1,140	1,309	−169	−12.9	33.1	37.0	−3.9	−10.5
7	1,335	1,401	−66	−4.7	34.2	37.0	−2.8	−7.6
8	1,396	1,392	4	0.2	35.7	37.0	−1.3	−3.5
9	1,256	1,287	−31	−2.3	36.6	37.0	−0.4	−1.1
10	1,493	1,416	77	5.5	36.4	36.5	−0.1	−0.3
11	1,105	1,196	−91	−7.7	34.3	36.5	−2.2	−6.0
12	1,346	1,405	−59	−4.3	33.1	36.5	−3.4	−9.3
13	1,580	1,576	4	0.1	34.1	36.5	−2.4	−6.6
14	1,457	1,513	−56	−3.8	33.7	36.0	−2.3	−6.6
15	1,457	1,417	40	4.2	34.9	36.0	−1.1	−3.0
16	1,331	1,423	−92	−6.7	32.5	36.0	−3.5	−9.7
17	1,218	1,218	0	0.0	34.2	36.0	−1.8	−5.0
Average..				−4.1				−6.5

* Surface area by the Du Bois height-weight chart.

In Table IV the averages of all observations on our subjects are compared with these two standards. Two of our subjects average higher than Benedict's standards, three almost exactly the same, and twelve lower. The general average is distinctly lower—4.1 per cent. In comparison with the Du Bois standard all our subjects without exception are low, ranging from −0.3 per cent to −13.8 per cent. Two are more than 10 per cent below the Benedict standard and three more than 10 per cent below the Du Bois.

We have no explanation of the low metabolism of our women. Most of them were leading fairly active lives, doing laboratory work, and some of them a limited amount of housework. They were probably not quite so active as the group of nurses studied by Harris and Benedict (9) and Palmer, Means, and Gamble (11) who also showed a metabolism below the standard. It is interesting, though of course not conclusive, that a number of people who were asked which of our group they considered most muscular all mentioned first the two individuals whose metabolism is above Benedict's standard.

Pulse Rate.

In connection with the metabolism determinations, data were collected on the pulse rate of the seventeen subjects, a total of 186 observations. They are included in Table VI and summarized in Table V. The counts were made during the experimental metabolism period after the subject had been lying down 35 minutes or more and may therefore be taken as minimum values. The averages for the different women range from 60 to 79. The average of these individual averages is 68.9, which is in rather surprisingly close agreement with that for Benedict's (9) 90 women—68.67, thus confirming his conclusion that pulse rate is higher in women than in men (Benedict's average 61.26) although basal metabolism is lower. The range of our single observations is from 54 to 100, or omitting this one extremely high case, from 54 to 88. Benedict's range is from 51 to 92.

There is no relation between pulse rate and basal metabolism for our different women (a lack of correlation also observed by Benedict), and, more surprising, there is no relation between the daily variation in basal metabolism and pulse rate for the single individual (a confirmation of Zuntz's (4) observations). The higher pulse rates do not occur on the same days as the higher basal metabolism nor the lower pulse rates as the lower metabolism. The correlation between pulse rate and metabolism which is often spoken of is their similar increase above minimum values brought about, for instance, in exercise (12), rather than the day by day variation in minimum. We have one exception to this statement of lack of agreement—the case of Subject 3, who in one day showed the exceptional pulse rate of 100, which is 30 beats above her next count, and also her highest basal metabolism, which was 11 per cent above her average and 19.6 per cent above her minimum. This 1 day thus shows an association of tachycardia and increased metabolism similar to that recently observed by Sturgis and Tompkins (13) in hyperthyroidism. It must be remarked, however, that even this maximum metabolism of our subject is only 3.1 per cent above Benedict's prediction for her. It is to be regretted that the body tempera-

TABLE V.

Subject.	Pulse.		
	No. of observations	Range.	Average.
1	25	54- 68	62
2	12	64- 78	68
3	7	64-100	74
4	5	72- 84	79
5	22	64- 84	71
6	10	56- 78	66
7	13	68- 88	79
8	10	56- 72	65
9	11	68- 80	75
10	12	64- 78	72
11	8	62- 66	65
12	5	60- 68	65
13	10	56- 80	67
14	9	60- 68	65
15	1	60	60
16	7	68- 78	72
17	19	64- 72	67
	Total...186		Average...68.9

TABLE VI.

Fundamental Data.

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day	Per kilo per hr.	Per sq. m. per hr.
	1920			*cc.*	*calories*	*calories*	*calories*
1	Jan. 27	Intermenstrual.	54	176	1,220	0.871	31.4
21 yrs.	Feb. 3	"	60	184	1,280	0.909	32.8
160 cm.	" 4	"	62	183	1,270	0.906	32.7
58.5 kg.	" 5	"	62	189	1,315	0.945	33.8
1.62 sq. m.	" 6	"	60	185	1,285	0.915	33.0
	" 7	"	58	179	1,240	0.902	32.0
	" 8	"	58	173	1,200	0.856	30.9
	" 9	"	60	184	1,280	0.909	32.8
	" 10	"	60	177	1,230	0.877	31.6
	" 11	"	62	183	1,270	0.906	32.7
	" 12	"	70	173	1,200	0.856	30.9
	" 13	"	60	178	1,235	0.880	31.8
	" 16	"	62	162	1,125	0.802	28.9
	" 17	"	66	178	1,235	0.880	31.8
	" 18	"	58	175	1,215	0.865	31.2
	" 19	"	66	177	1,230	0.877	31.6
	" 21	"	64	173	1,200	0.856	30.9
	" 22	"	58	166	1,155	0.822	29.7
	" 25	"	66	170	1,180	0.840	30.3
	" 26	"	68	176	1,220	0.871	31.4
	" 27	"	68	178	1,235	0.880	31.8
	" 28	"		188	1,310	0.932	33.7
	Mar. 1	"	60	173	1,200	0.856	30.9
	" 2	"	68	190	1,320	0.940	34.0
	" 4	"	64	185	1,285	0.915	33.0
	" 5	"	68	184	1,280	0.909	32.8
Average....			62		1,239		31.9
2	July 24	Intermenstrual.	64	204	1,420	0.888	36.3
24 yrs.	" 25	"		199	1,380	0.864	35.4
152 cm.	" 27	Premenstrual.		198	1,375	0.859	35.2
66.7 kg.	" 28	"	72	200	1,390	0.867	35.5
1.63 sq. m.	" 29	"	72	201	1,395	0.872	35.7
	" 30	"	72	200	1,390	0.867	35.5
	" 31	"	68	195	1,355	0.845	34.6
	Aug. 1	Menstrual.	64	216	1,500	0.935	38.3
	" 2	"	60	196	1,360	0.850	34.8
	" 3	"	72	207	1,440	0.898	36.8

TABLE VI—*Continued.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			*cc.*	*calories*	*calories*	*calories*
	Aug. 4	Menstrual.	68	207	1,440	0.898	36.8
	" 5	"	78	207	1,440	0.898	36.8
	" 6	"	66	214	1,490	0.930	38.1
	" 24	Intermenstrual.	64	204	1,420	0.888	36.3
Average....			68		1,413		36.2
3	Apr. 18	Intermenstrual.	70	176	1,220	1.033	34.6
25 yrs.	" 25	"		174	1,210	1.020	34.3
158 cm.	June 11	"	100	194	1,345	1.134	38.1
49.4 kg.	Aug. 8	"		170	1,180	0.995	33.4
1.47 sq. m.	" 9	"	66	173	1,200	1.017	34.1
	" 10	"	66	162	1,125	0.949	31.9
	" 11	"		180	1,250	1.055	35.5
	" 12	"	64	164	1,140	0.962	32.3
	" 14	"	64	172	1,195	1.006	33.8
	" 15	"	66	170	1,180	0.995	33.4
Average ..			74		1,205		34.2
4	Aug. 25	Premenstrual.	84	198	1,375	0.893	33.7
25 yrs.	" 27	Menstrual.	84	195	1,355	0.879	33.2
164 cm.	" 28	"	76	196	1,360	0.885	33.4
64.1 kg.	" 29	"	72	184	1,280	0.829	31.3
1.70 sq. m.	Sept. 2	Intermenstrual.	80	182	1,265	0.823	31.0
	" 3	"		186	1,290	0.840	31.6
Average...			79		1,321		32.3
5	Feb. 6	Intermenstrual.	72	195	1,355	1.125	37.3
26 yrs.	" 19	Premenstrual.	72	198	1,375	1.143	37.9
161 cm.	" 20	"	66	198	1,375	1.143	37.9
50.1 kg.	" 21	Menstrual.	72	187	1,300	1.081	35.9
1.51 sq. m.	" 22	"		200	1,390	1.153	38.3
	" 23	"	72	191	1,330	1.103	36.9
	" 24	"	64	177	1,230	1.023	34.0
	" 25	Postmenstrual.	70	186	1,290	1.074	35.7
	" 26	"	72	176	1,220	1.018	33.7
	" 28	Intermenstrual.	72	193	1,340	1.112	36.9
	Mar. 21	Premenstrual.	78	200	1,390	1.153	38.3
	" 23	"		197	1,370	1.139	37.7

TABLE VI—*Continued.*

Subject.	Date.	Phase.	Pulse rate	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			*cc.*	*calories*	*calories*	*calories*
	Mar. 24	Menstrual.		178	1,235	1.028	34.1
	" 25	"	72	172	1,195	0.992	32.9
	" 26	"	72	172	1,195	0.992	32.9
	" 27	"	68	191	1,330	1.103	36.9
	Apr. 10	Intermenstrual.	72	179	1,240	1.035	34.3
	Aug. 17	"	76	181	1,260	1.045	34.7
	" 18	"	78	174	1,210	1.005	33.4
	" 20	Premenstrual.	84	168	1,170	0.970	32.2
	" 21	"	68	158	1,100	0.912	30.3
	" 22	"	68	163	1,135	0.941	31.2
	" 23	Menstrual.	64	160	1,110	0.924	30.6
	" 24	"	78	165	1,145	0.954	31.7
	" 25	"	64	155	1,080	0.894	29.7
Average....			71		1,254		34.7
6	July 18	Menstrual	64	173	1,200	0.945	34.8
28 yrs.	" 20	"		157	1,090	0.857	31.5
148 cm.	" 21	"	60	154	1,070	0.842	31.0
53 kg.	" 22	"		162	1,125	0.886	32.6
1.44 sq. m.	Aug. 3	Intermenstrual.	72	160	1,110	0.873	32.1
	" 5	"	66	169	1,175	0.922	33.9
	" 6	"		172	1,195	0.939	34.5
	" 7	"	66	155	1,080	0.846	31.1
	" 12	Premenstrual.	78	173	1,200	0.945	34.8
	" 13	"		165	1,145	0.903	33.2
	" 14	Menstrual.	60	174	1,210	0.951	35.0
	" 15	"	56	158	1,100	0.862	31.7
	" 16	"	64	164	1,140	0.895	33.0
	" 17	"	72	162	1,125	0.886	32.6
Average....			66		1,140		33.1
7	Aug. 1	Menstrual.	68	200	1,390	0.948	35.5
28 yrs.	" 2	"	88	189	1,310	0.898	33.6
160 cm.	" 3	"	78	189	1,310	0.898	33.6
61.0 kg.	" 15	Intermenstrual.	80	200	1,390	0.948	35.5
1.63 sq. m.	" 16	"	72	191	1,330	0.906	33.8
	" 17	"	80	193	1,340	0.915	34.2
	" 26	Premenstrual.	84	199	1,380	0.944	35.3

TABLE VI—*Continued.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m per hr.
	1920			*cc.*	*calories*	*calories*	*calories*
	Aug. 27	Premenstrual.	80	196	1,360	0.930	34.8
	" 28	"	84	190	1,320	0.903	33.7
	" 29	"	80	190	1,320	0.903	33.7
	" 30	Menstrual.	76	187	1,300	0.889	33.2
	" 31	"	80	186	1,290	0.883	33.0
	Sept. 1	"	76	189	1,310	0.898	33.6
Average....			79		1,335		34.2
8 28 yrs. 161 cm. 59.6 kg. 1.63 sq. m.	May 24	Intermenstrual.	68	196	1,360	0.951	34.8
	" 25	"	64	212	1,470	1.033	37.8
	" 26	"	68	196	1,360	0.951	34.8
	" 27	"	56	209	1,455	1.017	37.2
	" 28	"	68	197	1,370	0.956	35.0
	" 29	"	56	195	1,355	0.945	34.6
	" 30	"	65	215	1,495	1.043	38.2
	" 31	"	72	197	1,370	0.956	35.0
	June 1	"	68	200	1,390	0.971	35.5
	" 2	"	66	193	1,340	0.936	34.2
Average....			65		1,396		35.7
9 29 yrs. 149 cm. 51 kg. 1.43 sq. m.	July 28	Premenstrual.	78	180	1,250	1.021	36.5
	" 29	Menstrual.	68	188	1,310	1.069	38.1
	" 30	"	76	181	1,260	1.028	36.6
	" 31	"	68	183	1,270	1.040	37.0
	Aug. 25	Premenstrual.	80	177	1,230	1.005	35.9
	" 26	Menstrual.	76	182	1,265	1.033	36.8
	" 27	"	72	186	1,290	1.055	37.6
	" 28	"	80	178	1,235	1.009	36.0
	Sept. 8	Intermenstrual.	80	177	1,230	1.005	35.9
	" 9	"	78	182	1,265	1.033	36.8
	" 10	"	72	176	1,220	1.003	35.8
Average....			75		1,256		36.6
10 30 yrs. 170 cm. 61.7 kg. 1.70 sq. m.	Mar. 11	Premenstrual.	78	222	1,540	1.043	37.9
	" 12	"	72	218	1,515	1.022	37.1
	" 13	Menstrual.	72	214	1,490	1.007	36.5
	" 14	"	68	205	1,425	0.962	34.9
	" 15	"	68	211	1,465	0.990	36.0

TABLE VI—*Continued.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production. Per day.	Heat production. Per kilo per hr.	Heat production. Per sq. m. per hr.
	1920			*cc.*	*calories*	*calories*	*calories*
	Mar. 16	Menstrual.	68	213	1,475	0.998	36.2
	June 25	Premenstrual.	76	228	1,585	1.068	38.8
	" 26	"	72	205	1,425	0.962	34.9
	" 27	Menstrual.	64	231	1,600	1.082	39.3
	" 29	"	72	204	1,420	0.958	34.8
	" 30	"	76	202	1,400	0.946	34.4
	Sept. 2	Intermenstrual.		225	1,560	1.055	38.3
	" 3	"	78	218	1,515	1.022	37.1
Average....			72		1,493		36.4
11	July 25	Intermenstrual.	64	165	1,145	1.112	35.7
32 yrs.	" 26	"	64	157	1,090	1.057	33.9
151 cm.	Aug. 4	Premenstrual.	66	162	1,125	1.091	35.0
43 kg.	" 5	"	66	159	1,105	1.072	34.4
1.34 sq. m.	" 6	Menstrual.	66	156	1,085	1.050	33.7
	" 7	"	62	147	1,020	0.988	31.7
	" 8	"	66	159	1,105	1.072	34.4
	" 23	Intermenstrual.	62	168	1,170	1.130	36.2
Average....			65		1,105		34.3
12	July 11	Menstrual.	66	190	1,320	0.887	32.6
33 yrs.	" 12	"	64	188	1,310	0.879	32.2
168 cm.	" 13	"	66	199	1,380	0.930	34.1
62 kg.	" 24	Intermenstrual.	60	206	1,430	0.962	35.3
1.69 sq. m.	" 25	"	68	186	1,290	0.868	31.9
Average....			65		1,346		33.1
13	Apr. 14	Intermenstrual.		239	1,660	0.875	35.8
33 yrs.	" 15	"		229	1,590	0.839	34.3
173 cm.	" 16	"	72	237	1,650	0.869	35.5
79 kg.	" 27	Premenstrual.	80	237	1,650	0.869	35.5
1.93 sq. m.	" 28	Menstrual.	72	233	1,620	0.855	35.0
	" 29	"	72	219	1,520	0.802	32.8
	" 30	"	72	233	1,620	0.854	35.0
	May 1	"	64	218	1,515	0.800	32.7
	" 25	Premenstrual.		228	1,585	0.834	34.2
	" 26	Menstrual.	56	227	1,575	0.832	34.1

TABLE VI—*Continued.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1920			*cc.*	*calories*	*calories*	*calories*
	May 27	Menstrual.	64	221	1,535	0.810	33.2
	" 28	"	64	221	1,535	0.810	33.2
	" 29	"	56	214	1,490	0.785	32.1
Average....			67		1,580		34.1
14 37 yrs. 162 cm. 75.5 kg. 1.80 sq. m.	July 22	Premenstrual.	60	200	1,390	0.766	32.1
	" 23	Menstrual.	64	209	1,455	0.804	33.7
	" 24	"	68	200	1,390	0.766	32.1
	" 25	"	64	209	1,455	0.804	33.7
	Aug. 10	Intermenstrual.	66	214	1,490	0.823	34.4
	" 11	"	64	217	1,510	0.832	34.9
	" 19	Premenstrual.	66	210	1,460	0.806	33.8
	" 20	Menstrual.	64	213	1,480	0.817	34.2
	" 21	"	66	214	1,490	0.823	34.4
Average....			65		1,457		33.7
	1919						
15 40 yrs. 166 cm. 66.6 kg. 1.74 sq. m.	Dec. 4	Intermenstrual.	60	216	1,500	0.936	35.8
	" 5	"		218	1,520	0.948	36.3
	" 12	Premenstrual.		203	1,410	0.884	33.8
	" 14	Menstrual.		205	1,425	0.891	34.1
	" 15	"		210	1,460	0.912	34.9
	" 16	"		205	1,425	0.891	34.1
Average....			60		1,457		34.9
	1920						
16 41 yrs. 158 cm. 70 kg. 1.71 sq. m.	Aug. 19	Intermenstrual.	72	196	1,360	0.811	33.2
	" 20	"	68	192	1,335	0.794	32.5
	" 21	"	78	190	1,320	0.789	32.3
	" 22	Premenstrual.		186	1,290	0.769	31.4
	" 23	"	72	196	1,360	0.811	33.2
	" 24	Menstrual.		197	1,370	0.814	33.4
	" 25	"	72	180	1,250	0.745	30.5
	" 26	"	72	190	1,320	0.789	32.3
	" 27	"	72	198	1,375	0.819	33.5
Average....			72		1,331		32.5

TABLE VI—*Concluded.*

Subject.	Date.	Phase.	Pulse rate.	Oxygen per min.	Heat production.		
					Per day.	Per kilo per hr.	Per sq. m. per hr.
	1919			*cc.*	*calories*	*calories*	*calories*
17	Dec. 3	Premenstrual.	68	182	1,265	1.072	35.4
44 yrs.	" 4	"	72	172	1,195	1.011	33.4
162 cm.	" 5	"	64	183	1,270	1.080	35.6
49.1 kg.	" 6	"	68	189	1,315	1.113	36.7
1.49 sq. m.	" 8	"	72	165	1,145	0.974	32.1
	" 9	Menstrual.	70	189	1,315	1.113	36.7
	" 11	"		183	1,270	1.080	35.6
	" 12	"		187	1,300	1.103	36.4
	" 13	Postmenstrual.		181	1,260	1.068	35.2
	" 17	Intermenstrual.	72	185	1,285	1.090	35.9
	" 19	"	68	167	1,160	0.985	32.4
	1920						
	Mar. 7	"	66	170	1,180	1.011	33.3
	" 15	Premenstrual.	64	155	1,080	0.922	30.3
48.7 kg.	" 16	Menstrual.	64	164	1,140	0.975	32.1
1.48 sq. m.	" 17	"		164	1,140	0.975	32.1
	" 19	"	68	176	1,220	1.045	34.4
	" 20	"	72	178	1,235	1.057	34.8
49.5 kg.	Apr. 7	Premenstrual.	70	176	1,220	1.028	33.9
1.50 sq. m.	" 8	"		180	1,250	1.061	34.7
	" 9	"		181	1,260	1.058	34.9
	" 10	"	60	171	1,190	1.000	33.0
	" 11	Menstrual.	64	178	1,235	1.041	34.4
	" 12	"		176	1,220	1.028	33.9
	" 13	"		176	1,220	1.028	33.9
	" 14	"		169	1,175	0.987	32.6
	" 27	Intermenstrual.	72	171	1,190	1.000	33.0
	" 29	"	64	169	1,175	0.987	32.6
	" 30	"	64	181	1,260	1.058	34.9
	May 1	"		169	1,175	0.987	32.6
Average....			67		1,218		34.2

ture was not determined on this day. None of our other pulse rates is within the range of pathological significance.

The variation of pulse rate during menstruation is almost as irregular as the basal metabolism. In three cases Subjects 2, 7 (first period observed), and 10 (first period observed), there is a slightly higher pulse rate the first of the menstrual period than later, an observation in agreement with King (14), who found that pulse rate and temperature follow the rhythmical movement in life processes, while blood pressure, systolic and diastolic, and pulse pressure varied irregularly. In most of our subjects, however, no regular variation was observed.

SUMMARY.

1. Series of 216 observations are given on the basal metabolism of 17 women, 14 of them including 1 or more menstrual cycles, and 1 being observed for 26 almost consecutive days.
2. There is no definite change in the basal metabolism during menstruation. This is seen from the facts that the average of the intermenstrual and menstrual observations is almost the same, and that no rhythmical periodic variation in metabolism can be noted.
3. The daily variation for each subject is great, ranging from 7.4 to 28.8 per cent, or an average of 13.2 per cent. This is slightly less than the average which Benedict found, 13.9 per cent, with a group of individuals observed 5 days or more. Erroneous conclusions can easily be drawn from metabolism observations unless measurements are made on more than 1 day.
4. Most of our subjects show a somewhat lower basal metabolism than that calculated for them from the Benedict or the Du Bois standards.
5. There is no relation between minimum pulse rate and basal metabolism in our subjects, except in one case for 1 day where the pulse rate increased to an extent which may be considered pathological. Neither is there definite constant change in the pulse rate during menstruation.

The authors wish to express their thanks to Dr. F. G. Benedict for reading and criticizing the manuscript of this paper, to Dr. T. M. Carpenter for helpful suggestions at the beginning of the work, and to the women who served as subjects, through whose interest and cooperation the investigation was made possible.

BIBLIOGRAPHY.

1. Benedict, F. G., A portable respiration apparatus for clinical use, *Boston Med. and Surg. J.*, 1918, clxxviii, 667.
2. Benedict, F. G., Notes on the use of the portable respiration apparatus, *Boston Med. and Surg. J.*, 1920, clxxxii, 243.
3. Du Bois, D., and Du Bois, E. F., Clinical calorimetry. X. A formula to estimate the approximate surface area if height and weight be known, *Arch. Int. Med.*, 1916, xvii, 863.
4. Zuntz, L., Untersuchungen über den Einfluss der Ovarien auf den Stoffwechsel, *Arch. Gynäk.*, 1906, lxxviii, 106.
5. Gephart, F. C., and Du Bois, E. F., Clinical calorimetry. XIII. The basal metabolism of normal adults with special reference to surface area, *Arch. Int. Med.*, 1916, xvii, 902.
6. Snell, A. M., Ford, F., and Rowntree, L. G., Studies in basal metabolism, *J. Am. Med. Assn.*, 1920, lxxv, 515.
7. Boothby, W. M., The fundamental classification of disease by the basal metabolic rate, *J. Am. Med. Assn.*, 1921, lxxvi, 84.
8. Benedict, F. G., Factors affecting basal metabolism, *J. Biol. Chem.*, 1915, xx, 263.
9. Harris, J. A., and Benedict, F. G., A biometric study of basal metabolism in man, *Carnegie Inst. Washington, Pub. 279*, 1919.
10. Aub, J. C., and Du Bois, E. F., The basal metabolism of old men, *Arch. Int. Med.*, 1917, xix, 823.
11. Palmer, W. W., Means, J. H., and Gamble, J. L., Basal metabolism and creatinine elimination, *J. Biol. Chem.*, 1914, xix, 239.
12. Benedict, F. G., and Murchhauser, H., Energy transformations during horizontal walking, *Carnegie Inst. Washington, Pub. 231*, 1915.
13. Sturgis, C. C., and Tompkins, E. H., A study of the correlation of the basal metabolism and pulse rate in patients with hyperthyroidism, *Arch. Int. Med.*, 1920, xxvi, 467.
14. King, J. L., Concerning the periodic cardiovascular and temperature variations in women, *Am. J. Physiol.*, 1914, xxxiv, 203.

FAT-SOLUBLE VITAMINE.

VII. THE FAT-SOLUBLE VITAMINE AND YELLOW PIGMENTATION IN ANIMAL FATS WITH SOME OBSERVATIONS ON ITS STABILITY TO SAPONIFICATION.*

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When in the early part of 1919, we (1, 2) formulated the working hypotheses, in our fat-soluble vitamine investigation, that the vitamine might be identical with, or closely related to certain yellow pigments of the carotinoid type, we made the attempt to correlate its occurrence on this basis, to formulate a procedure for its isolation, and to collect information as to its possible chemical nature. In these objects we have been substantially aided by our hypotheses, for while all the possibilities anticipated have not materialized yet there has been given a direction to our experimental efforts in this field, and apparently to those of others, which has lead to great centralization of effort. It was obvious that, in spite of the numerous instances of association of the physiological growth-promoting property which is attributed to the presence of fat-soluble vitamine and yellow plant pigments, the two would not necessarily have to be identical, in fact there need be no material relationship in composition or structure, as their coincident occurrence in nature might be due to physiological determination, pure and simple. In this event, with the diversification of metabolic processes which obtain in the plant and animal kingdom, it was to be expected that sooner or later the fat-soluble vitamine would be found to be present in a menstruum entirely free from pigments of the carotinoid type. To run across such an instance, appears to

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have been the good fortune of Palmer and Kempster (3), who demonstrated that pork liver, rich in the vitamine, contained no pigments of the aforementioned character. Gross of this laboratory has obtained similar results[1] but Rosenheim and Drummond (4) purport to have shown that while carotin and xanthophyll are absent, another pigment of this type is present. It may still be said that in such instances where the fat-soluble vitamine is found to occur in the absence of color the pigment is present in the leuco form (2). While this can be accepted as a possibility it is at present not worthy of serious consideration because nothing is known of the structure of the carotinoid molecules and therefore nothing is known as to the probability of the existence of leuco compounds in the series. Nevertheless, as far as studies in this domain have been pursued, both in regard to distribution of vitamine and pigment and in regard to their physical and chemical properties, there is left no doubt but that chemically and physiologically they are related. These relations will be discussed in succeeding papers in which our results will be correlated with those of others who have been active in this field of investigation. In this paper it is desired to present data on the fat-soluble vitamine content of various animal fats as correlated with yellow pigmentation, and to record a few observations made on its stability to saponification.

EXPERIMENTAL.

For the laboratory technique employed in these experiments the reader is referred to previous papers of this series (5–10), as the experimental methods employed were essentially the same. In no instance has it been deemed permissible to make any selection of data. Four animals, usually two males and two females, were started in each group and continued until death or until the differences among the groups became so pronounced as to make further continuation superfluous. In a few instances where data were purely confirmatory we have felt at liberty to to present merely representative illustrations.

Failure of growth alone has not been accepted by us as a good criterion to use in establishing the lack of the fat-soluble vitamine and this for very obvious reasons. Emphasis was, therefore, always placed upon the appearance of the eyes of the rats, as an inflamed condition, an ophthalmia variously termed a conjunctivitis, a xerophthalmia, and a keratomalacia, makes its appearance in the vast majority of animals on a fat-soluble vitamine-poor diet as first pointed out by Osborne and Mendel (11). Though the exact condition which is incident when the eyes become infected is not described accurately by these terms we have used them rather indiscriminately in the past hoping that pathologists would soon describe the condition in detail and designate it accurately.[2] Stephenson and Clark (12) introduced the term keratomalacia in preference to xerophthalmia, apparently because in these inflammatory reactions a softening of the cornea with loss of the lens frequently results. In a certain sense each of these terms has its proper application in the various types or stages of the ophthalmitis observed. Generally a conjunctivitis with an erythema and edema of the eyelids appears first, later a keratitis or inflammation of the cornea results, and ultimately, if the diet is not corrected or if resistance to the infection does not develop of its own accord especially in severely purulent inflammatory reactions, a dryness or xerosis of the eye—a xerophthalmia—marks the culmination of the eye symptoms. As usually observed and especially in the primary stages the condition is marked by an excessive secretion and is certainly not a xerophthalmia. We believe that, in most instances when the inflammation is not confined to the conjunctiva, the infection is most accurately designated as a keratoconjunctivitis. Only occasionally will we have reason to speak of a xerophthalmia because true xerophthalmia has rarely made its appearance in our colony. When the inflammatory reaction is so severe as to cause permanent injury and the diet is not corrected, death results rapidly; if it is transitory, making its appearance now and then—indicative of only a partial deficiency of the fat-soluble vitamine—then practically complete recovery has always been observed. The recovery cannot be considered entirely complete because an inflamed condition of the eyes, beyond a mere infiltration of fluid—an edema of the conjunctiva and eyelids—leaves its permanent mark in the destruction of the hair follicles on the lids. This "bare eyed" condition always arouses our suspicions of a fat-soluble vitamine deficiency, suggesting that a transitory reaction has escaped our notice if the inflammation was not actually observed.

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[1] Unpublished data.

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[2] Such studies have recently been published by Wason (Wason, I. M., *J. Am. Med. Assn.*, 1921, lxxvi, 908), since the preparation of this manuscript.

In addition to the inflammation of the eyes, we have observed in many animals, large and small, an apparent resistance to infection of the eyes even though continually exposed by contact to severely infected animals, all on a fat-soluble vitamine-free ration. In such individuals an enophthalmia or a "small eyed condition" is frequently observed. The eyeballs are not "beaded" as in normal rats but appear small and sunken in the orbital cavity. Many of these individuals succumb to respiratory infections, in fact we have been led to think that possibly a certain immunity to infection of the eyes is thus conferred.

The incidence of respiratory infections as part of the syndrome induced by fat-soluble vitamine deficiency was described by McCollum (13) in his early work. It may consist, as we have observed, of a nasal or bronchial catarrh or even pulmonary infection with mucous or purulent exudate, at times even resulting in hemorrhage. Animals thus afflicted in the early stages of the disease sneeze and cough violently but later as the inflammation becomes confined more to the lungs the cough subsides and dyspnea becomes very pronounced with the slightest activity. Such animals fail very rapidly and even with the introduction of fat-soluble vitamine in the ration rarely show normal growth subsequently.

A fat-soluble vitamine deficiency is also far from being conducive to normal cutaneous nutrition so that very often—especially after an age of 4 months has been reached—evidence of dermal malnutrition makes its appearance. The fur appears bushy and thin, cutaneous growths occur on the tail, ears, and nose, and finally sores, which heal with difficulty, appear on the feet, limbs, and body; all bear testimony to this state of malnutrition.

All of the aforementioned symptoms and conditions have been carefully watched and noted at the time of the weekly weighings of our animals unless their condition or the nature of the experiment made examinations at shorter time intervals of moment. Judgment was based on the sum total of indications.

Fat-Soluble Vitamine Content of Cod Liver Oil.

Since Osborne and Mendel (14) in their pioneering studies on the fat-soluble vitamine found cod liver oil to contain this dietary essential surprisingly little experimental endeavor has been made to study it in its quantitative relations though repeated emphasis has been placed on the efficacy with which it can be used as a therapeutic agent in perverted metabolism of the osteoid tissues. As far as known to the writers, Zilva and Miura (15) are the only investigators who have studied this problem. They have recently published a preliminary note on their experiments stating that cod liver oil in its crude state was found to be 250 times as potent as butter in furnishing this constituent.

Our investigations were not outlined to bring out such extreme differences as we were primarily intent on a correlation of the fat-soluble vitamine with pigment content in comparison with the intense pigmentation of butter, and selected cod liver oil because we had at hand an excellent sample which in comparison with butter was practically devoid of yellow pigments—it had only a faint yellowish green color. If the vitamine were a yellow pigment it should therefore have shown only limited activity. The sample of oil was prepared from fresh cod livers with minimum heat exposure. The livers were cut into small pieces, put into a steam-jacketed open cooker, and heated not to exceed 180° F. The oil collecting on the surface was skimmed off as rapidly as it formed in the first 35 minutes of heating, filtered through paper, and bottled.

As seen in Chart 1, this oil when fed at a level corresponding to 2 and 5 gm. of material in a kilo of ration was exceedingly active; it was far more efficient than any sample of butter fat that we have ever studied. This alone was one of the things which made us very skeptical in the early course of our studies of the assumption that the fat-soluble vitamine was necessarily a yellow pigment.

Seasonal Variation in the Fat-Soluble Vitamine Content of Butter.

Much has been written and said about the fat-soluble vitamine content of butter fat primarily because of its extensive use as an article of food and therefore its probable importance as a source of the fat-soluble vitamine in the human dietary. Due to the comparatively low intake of butter fat in the diet of the adult, the destruction of its vitamine in some cooking processes, the general occurrence of the vitamine in many foods such as green plant tissue, certain seeds and even roots, and the undetermined requirements of man, it is questionable whether its vitamine content would now attract the attention that it does if it were not for the fact that butter fat was the material in which the vitamine was first discovered. Early in our studies we were impressed with the fact that butters varied decidedly in their vitamine content; most of them being very rich in this dietary constituent, but some being as poor as the average oleomargarine (5). Certain observations which we made also impressed us with the lability of the vitamine as we found it destroyed in heated butters and in butters kept under poor storage conditions (5), so that we did not feel at liberty to draw conclusions with respect to the primary causal factors involved.

As our studies on the distribution of the fat-soluble vitamine progressed and indications of the occurrence of the vitamine with yellow pigments were obtained it appeared profitable to attempt to correlate these relations in butter fat especially in view of the fact, as is well known in dairy practice, that butter churned in late winter or early spring under Wisconsin conditions without artificial coloring is practically void of all color. If the vitamine content should be demonstrated to be of the same order of magnitude as the pigmentation then further presumptive evidence of this relation would have been obtained.

To this end there were prepared samples of butter fat in the latter part of the months of March, April, May, and June from cream obtained at the University creamery which was representative of the composite collection from a large number of dairy farms in the vicinity. The butter was churned in the laboratory, melted, and filtered at a low temperature, and then stored in a refrigerator till used in the experiments. The butter fat was fed in basal rations which have been repeatedly demonstrated to be very low in the fat-soluble vitamine content, though controls were never omitted. Originally, through the March series of experiments, the basal ration was one which we have used before, consisting of: casein, 18; agar, 2; Salts 32, 4; ether-extracted wheat embryo, 6; and dextrin, 70. In the April, May, and June series a white corn ration, consisting of: white corn, 40; casein, 14; Salts 32, 3; Salts 35, 1; and dextrin, 42; was used. This latter is an excellent ration and guarantees a sufficiency of the water-soluble vitamine being introduced with the white corn which is not always the case when a variable commercial product such as wheat embryo is used as its source. The butter fats of unknown value were introduced in these rations at the expense of so much dextrin.

As illustrated in Charts 2, 3, and 4 there occurs a decided variation in the vitamine content of the different butters; but even gross inspection of the monthly collections made it evident to us that the variations in vitamine were not quantitatively reconcilable with the variations in pigment. To enable more accurate comparisons to be made we availed ourselves of the use of a standard color solution in a Duboscq colorimeter, which was prepared by dissolving 7 gm. of K_2CrO_4 and 0.074 gm. of $K_2Cr_2O_7$ in water and making it up to 100 cc. volume. Such a solution compares favorably with the color of June butter fat but as the intensity of pigmentation is reduced, as is the result in the winter butter fat, the effect of a residual yellowish green pigmentation becomes disturbing and comparisons are not so easily made. Nevertheless, the accuracy of the determinations exceeded by far the requirements of our work as the results of the feeding trials themselves cannot be evaluated with any great degree of accuracy. With June butter fat accepted as having a value of 100, May butter fat was found to have a value of 86, and March and April butter fats, a value of 2.8. The latter were therefore practically colorless. With these factors in mind, upon inspection of the growth curves, it becomes increasingly evident that the fat-soluble vitamine content of the butter fats does not run parallel to the intensity of pigmentation; otherwise in the first place May and June butter fats fed at the 0.5 per cent levels should have been far more potent than they actually were in comparison with the March or April butter fats as they carried from 30 to 35 times as much pigment. In the second place when fed at different levels, 2 per cent for the March and April butter fats as compared with 0.5 per cent for the May and June butter fats, the former should not have exceeded the latter in efficiency as even then only from one-eighth to one-ninth as much total pigment had been introduced into the ration. These findings harmonize with those of Drummond and Coward (16) who arrived at similar conclusions.

Before the facts of these relations were obtained many attempts were made in the summer of 1919 to ascertain if any parallelism between vitamine and pigment content obtained by taking advantage of the fact that the carotin in butter fat is easily destroyed by heating. We heated butter fat in deep and in shallow dishes in the presence and absence of oleic acid—as acids accelerate pigment destruction in butter very markedly—with and without aeration with hydrogen, carbon dioxide, and air. We expected that if vitamine and pigment were not identical under some of these conditions destruction of the one without destruction of the other might be found to occur. Our results were entirely unsatisfactory as consistent duplication of results on different samples could not be obtained. As the selection of data bearing out a particular point at issue is not justifiable when unexplainable contradictory evidence is also obtained, the results of this work were not published. They served to convince us, however, that the success of such experimental attempts depended largely upon good fortune as butter fat is too variable in fat-soluble vitamine content to be taken as a good source of vitamine for studies of this character.

Since these experiments were carried out, Stephenson (17) has submitted data which tend to show that charcoal can be successfully employed in the removal or destruction of the pigment without causing complete destruction of the vitamine. Unfortunately her experimental period is shorter than desirable, especially as she worked with animals of considerable size in which we have found under normal conditions the vitamine reserve to be high and continued normal growth for 8 weeks to be common. The sudden death of one individual is not reassuring as pulmonary infections carry off some individuals on a fat-soluble vitamine-poor diet without premonitory symptoms in the course of a few days; nevertheless her data are very suggestive especially in view of Palmer's (3) observations on the feeding of pork liver.

Fat-Soluble Vitamine in Beet Fats.

Osborne and Mendel (18) and later Halliburton and Drummond (19) showed that beef fats might contain considerable amounts of the fat-soluble vitamine though in general their efficiency in furnishing this dietary constituent was not to be compared with butter fat. By fractionally crystallizing the beef fats from alcohol, Osborne and Mendel obtained a very active fraction. The beef oils were found to be exceedingly active while the solid residue was inactive. In our work a somewhat similar product, the oleo oils from beef fats, prepared in commerce for the manufacture of oleomargarine, were in some instances found richer in the fat-soluble vitamine than many butters (5). Subsequent to the publication of these results we became aware of the fact that the vitamine content as determined in our feeding experiments with these samples seemed to vary directly with the intensity of pigmentation. This led to the collection of additional data to determine if this was a mere coincidence or if it was commonly true.

During 1919 the experiments were confined to the investigation of the perinephric fat of animals of the Jersey, Durham, and Holstein breeds. The fatty tissue was ground in a meat hasher and extracted by heating slightly above the melting point in a steam oven and then straining and decanting the melted fats. They were preserved in Mason jars in a refrigerator until utilized in the experiments.

As seen in Chart 5, the Jersey fat was very active while the Durham fat gave no evidence of containing this vitamine. The same inactivity was shown by the Holstein fat. Both the Holstein and Durham fats were practically colorless; the Jersey fat, on the other hand was fully as pigmented as a sample of June butter.

In 1920 we duplicated these experiments except for the fact that the samples were not taken from any particular breeds but were selected promiscuously from slaughtered animals for color intensity. The dark beef fat was fully equal in color to June butter, the medium beef fat was two-thirds as colored and the light beef fat only one-tenth as colored. These values were obtained by measurement in a Duboscq colorimeter. The results shown in Charts 6 and 7 are essentially of the same character as those obtained the year before—the fat-soluble vitamine content roughly parallels the pigmentation. In view of the results that we have obtained with butter fat, it is not to be concluded that this is necessarily always the case. The rapidity of fat deposition, its mobilization, and the variation in the assimilation of pigment with different breeds and individuals, no doubt all operate to modify the primary determinative effect of the composition of the ration. Just how the latter may influence the relations we have again had occasion to observe with the fat-soluble vitamine content of egg yolks.[1] Normally, on ordinary rations light-colored yolks are low in the fat-soluble vitamine; yet by the selection of a special and unusual ration we have succeeded in producing light-colored yolks of normal vitamine content.

Stability of Fat-Soluble Vitamine to Saponification.

The study of the characteristics of the fat-soluble vitamine has presented considerable difficulties particularly due to mistaken notions of its stability and solubility properties which were fostered by suggestions rather than conclusive evidence as presented by various investigators. McCollum and Davis (20) reported the transference of the fat-soluble vitamine from butter fat into olive oil after the butter fat had been submitted to a mild saponification at room temperature. This was submitted as a preliminary paper in 1914 as it was stated that other experiments were under way and would be reported as soon as advisable. In the experiments detailed by them a number of difficulties can be appreciated which have made duplication very difficult as no confirmation of these attempts has been published. In the first place the drying of the soaps and the dissipation of the ether vapors from the ether-olive oil extract are processes not easily carried out under laboratory conditions without causing the destruction of considerable amounts of the vitamine. In the second place the olive oil extract as fed at a 3 per cent level and, therefore, equivalent to 6 per cent butter fat, did not leave a sufficient margin of fat-soluble vitamine to guarantee its presence at the close of operations as many samples of butter fat without having been subjected to any treatment are ineffective when fed at this level. In the experiments mentioned, however, it is possible that the 20 per cent lactose (21) carried considerable vitamine so that but a small increment was needed to elicit a growth response. Nevertheless, all these facts made it appear very unprofitable to attempt to repeat these experiments especially as the vitamine was ultimately brought into the solution of a fat with no determination of the completeness of the saponification beyond an inspection of the solubility of the reaction mixture which in the presence of so much soap is far from satisfactory. These experiments are therefore to be considered merely as a demonstration of the resistance of the fat-soluble vitamine to the mild saponification employed. In the light of this it was not surprising that Drummond (22) failed in demonstrating the resistance of the vitamine to the mild saponification of Henriques used by McCollum. He varied his procedure in that he attempted an ether extraction of the soaps but failed to show any activity of either the extract or an ether extract of the saponified residue when he fed the equivalent of 15 and 20 per cent of butter fat and whale oil, respectively.

From our work on the extraction of the vitamine from plant materials, where we adopted the method of separation in use for the carotinoids, we have demonstrated repeatedly the resistance of the vitamine to saponification and its subsequent extractibility by ether (10). From our present work (Chart 8), it is evident that the fat-soluble vitamine as found in animal fats has similar properties. In two instances, Lots 741 and 969, the saponification was conducted at 37°C. for 4 hours; 300 gm. of the fat being treated with 600 cc. of 20 per cent alcoholic potash, which are the proportions of fat and alkali used in the methods of analysis of The Association of Official Agricultural Chemists (23). At the end of the 4 hour period 2,400 cc. of water were added and the aqueous alcoholic solution of soaps extracted three times with ether. The ether extracts were washed with a small volume of water and then evaporated directly at room temperature in an air current on the ration. For control purposes a saponification was carried out with butter fat parallel to one run by the Official Methods using the same concentration and excess of alkali, acting for the same period of time at the same temperature as the preparation. They gave the same saponification value indicating that the saponification in our butter fat preparation was complete. Nevertheless, we made another preparation in which the fat was boiled with 20 per cent alcoholic potash for one-half hour as required by the Official Methods but with no reduction in alkali concentration. The growth curves of Lot 972 bear testimony to the fact that even under these drastic conditions the fat-soluble vitamine was not destroyed to any appreciable extent. From this it can be concluded that it is not a fat or an ester and that it is not labile to heat in the presence of a high concentration of alkali.

We desire to express our appreciation to Lord Brothers who furnished the cod liver oil and Armour and Company who furnished us with the beef fats.

SUMMARY.

In cod liver oil there is present a very high concentration of the fat-soluble vitamine with but small amounts of yellow pigments.

Butter fat shows a seasonal variation in the fat-soluble vitamine content when obtained from stall fed cows during the winter and pastured in the summer as is the practice under Wisconsin conditions.

The fat-soluble vitamine content of butter fat does not run closely parallel to the yellow pigment; yet in general, due to determination by their content in the feed, butters highly pigmented are rich in the vitamine; butters low in pigment should be looked upon with suspicion.

In beef fats the relations are somewhat similar; those most pigmented are also generally richest in their fat-soluble vitamine content.

The fat-soluble vitamine withstands severe methods of saponification. This indicates that it is not a fat and probably

not an ester and makes possible the compounding of satisfactory fat-free synthetic rations for investigative purposes.

BIBLIOGRAPHY.

1. Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1920, xli, p. xii.
2. Steenbock, H., *Science*, 1919, l, 353.
3. Palmer, L. S., and Kempster, H. L., *J. Biol. Chem.*, 1919, xxxix, 299.
4. Rosenheim, O., and Drummond, J. C., *Lancet*, 1920, i, 862.
5. Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.
6. Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.
7. Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81.
8. Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1920, xli, 149.
9. Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 163.
10. Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xlii, 131.
11. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 431.
12. Stephenson, M., and Clark, A. B., *Biochem. J.*, 1920, xiv, 502.
13. McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.
14. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 401.
15. Zilva, S. S., and Miura, M., *Lancet*, 1921, i, 323.
16. Drummond, J. C., and Coward, K. H., *Biochem. J.*, 1920, xiv, 668.
17. Stephenson, M., *Biochem. J.*, 1920, xiv, 715.
18. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.
19. Halliburton, W. D., and Drummond, J. C., *J. Physiol.*, 1917, li, 235.
20. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1914, xix, 245.
21. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231.
22. Drummond, J. C., *Biochem. J.*, 1919, xiii, 81.
23. *Bull. 107, Bureau of Chemistry, United States Dept. Agric.*

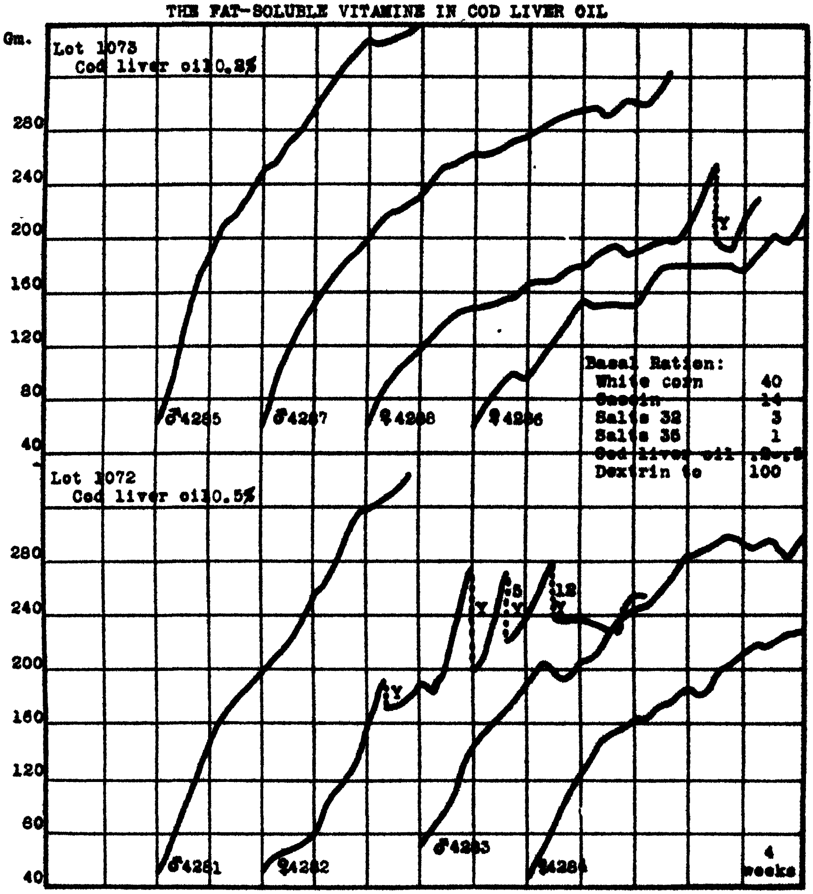

Chart 1. Lots 1072 and 1073 illustrate the remarkable growth-promoting property of small amounts of cod liver oil as a source of the fat-soluble vitamine. Rats 4285 and 4281 both continued their phenomenal rate of growth beyond the curves of growth shown here, the former weighing 395 gm. and the latter, 422 gm. 6 weeks later. Rat 4282 raised three young out of a litter of twelve in 5 weeks to an average weight of 40 gm. None of the other litters was raised. White Indian corn at a 40 per cent intake level was used as the source of water-soluble vitamine as it has been shown in numerous experiments to lead to nutritive failure as a source of the fat-soluble vitamine and yet it furnishes plenty of the water-soluble vitamine for normal growth.

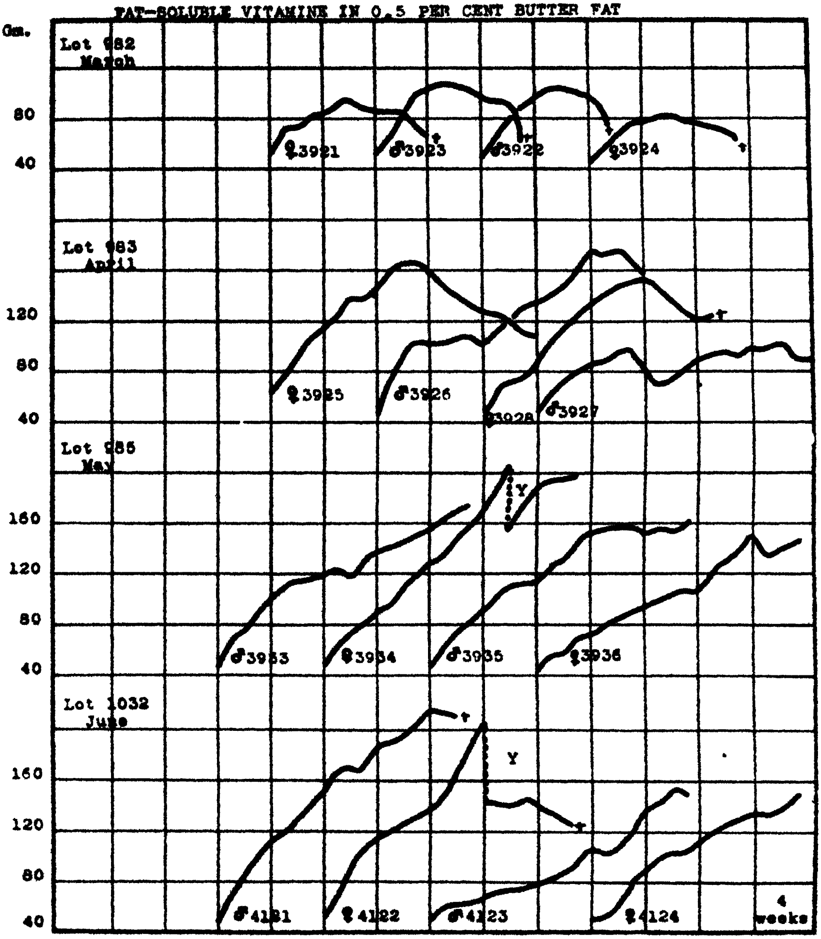

CHART 2. This chart shows the variable effects in growth responses when an attempt is made to introduce the fat-soluble vitamine into the ration by the substitution of 0.5 per cent of butter fat collected in successive months for 0.5 per cent of dextrin. On the March collection in Lot 982 by the pronounced failure of growth and even maintenance in all the animals, by the dermal infections of Rats 3921, 3922, and 3923 and by the ophthalmia in Rats 3922, 3923, and 3924, there is left no doubt that a deficiency of the fat-soluble vitamine obtained. On the April collection, Lot 983, growth was considerably better but all the animals were afflicted with keratoconjunctivitis. On the May butter fat, Lot 985, growth was continuous but dermal nutrition was poor; no eye symptoms were observed. On June butter fat, Lot 1032, the experimental results were practically the same except that dermal malnutrition was not evident, and Rat 4122 contracted a prolonged bronchial infection. Young were not reared.

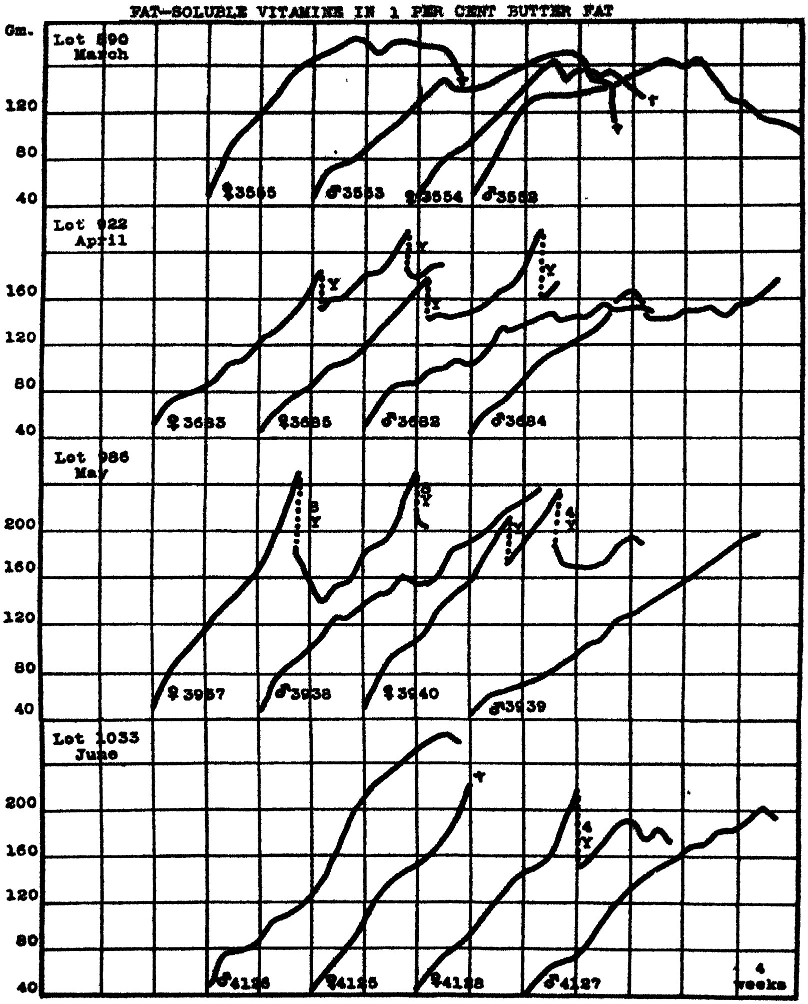

CHART 3. When the butter fat was increased to 1 per cent of the ration, growth was in all cases much improved with marked evidence of inferiority of the March product as fed in Lot 890. All of the rats contracted keratoconjunctivitis before their death and Rat 3553 by its sneezing and coughing indicated the presence of a respiratory infection. The April sample, although not up to par, was evidently richer in fat-soluble vitamine content, as the growth performance of the animals was much better and no indisputable symptoms of conjunctivitis were observed. There were, however, some indications of cutaneous malnutrition as the tails of the animals bore some infections towards the close of the experimental period. For May and June, Lots 986 and 1033, no special comments as to the normality of growth appear to be called for. On the May product Rat 3940 even raised a litter of four without apparent difficulty.

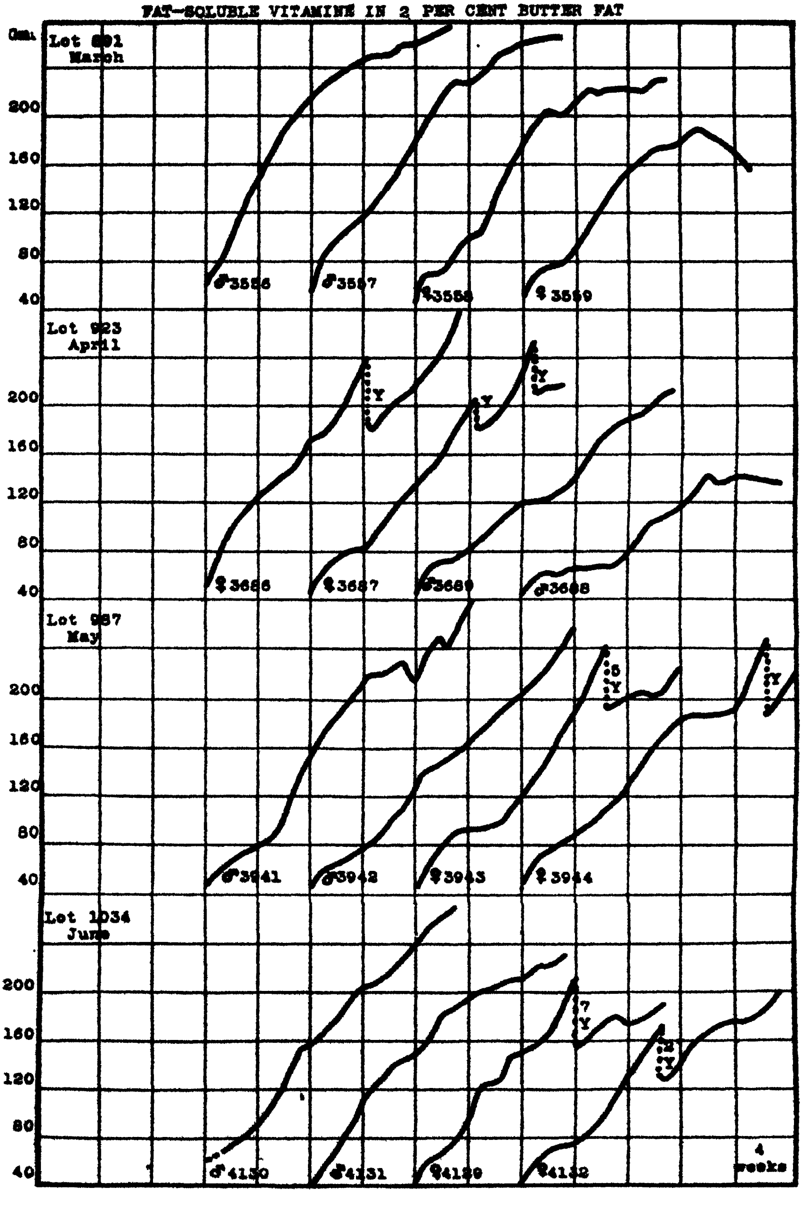

Chart 4. With the butter fat increased to 2 per cent of the ration deficiencies in the nutritive value of the collections, as far as evident were entirely eliminated, if we except the fact that Rat 3559 failed due to a localized caseous pulmonary infection. All other individuals remained normal.

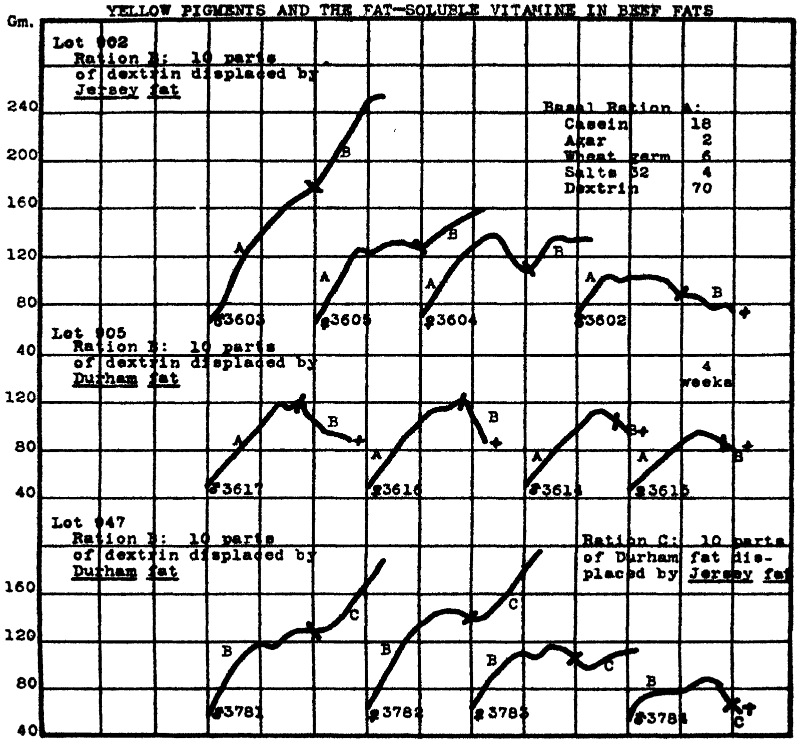

CHART 5. This chart illustrates the growth performance of rats on the perinephric fats of cattle as a source of the fat-soluble vitamine. In Lot 902, Rats 3602 and 3604 were afflicted with keratoconjunctivitis, the eyes of the others being normal when the change to Ration B carrying the Jersey fat was made. Except for Rat 3603 the condition of the animals was poor so that the response to the improvement of the diet was probably not of the order of magnitude to be expected. This is suggested by the results shown by Lot 947.

Lot 905. When the rats of this lot were changed to the fat-containing ration, all of the rats were affected with ophthalmia. The eyes of only Rat 3617 showed improvement subsequent to the change before death ultimately supervened. This was the only suggestion of the possible presence of the fat-soluble vitamine; possible because improvement of eyes is sometimes though not generally observed without any change having been made in the diet, yet growth in such cases is not restored and death slowly results.

Lot 947 when started out on a ration containing ten parts of Durham fat, such as was substituted in the ration of Lot 905, did not enable the rats to grow any longer or better than when the fat was not included. All of the rats showed inflamed eye conditions which promptly subsided shortly after the change to Jersey fat was made. This was true even in the case of Rat 3784 which however, due to its impoverished condition, died shortly thereafter.

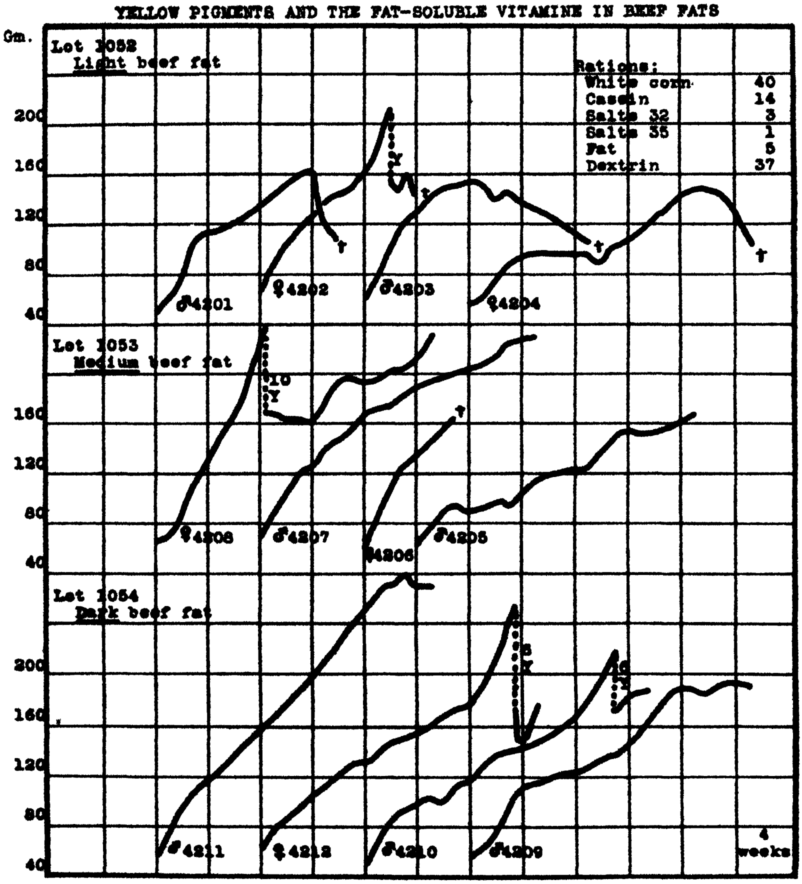

CHART 6. This chart shows the growth observed on the 1920 beef fat samples when the animals were given the fat addition from the beginning of the experiment. In Lot 1052, on the light beef fat samples, Rat 4201 developed pulmonary infections and Rats 4203 and 4204, keratoconjunctivitis. The appearance of Rat 4202 alone remained fairly normal but rapid failure ensued after parturition which is very often observed on a fat-soluble vitamine-poor ration. In Lot 1053 on the medium beef fat Rat 4205 contracted conjunctivitis after 16 weeks on the ration, later by the 20th week its eyes turned purulent with complete recovery by the 26th week, but by that time indications of dermal malnutrition were very distinct; it showed loss of hair, localized infections on body, and a horny epithelial growth on its nose. Rats 4208 and 4207 gave indications of dermal malnutrition only. These conditions in general suggest a deficiency in the fat-soluble vitamine content even though growth was fairly good. In Lot 1054 on the dark beef fat all the animals maintained themselves in good condition to the end of the experimental period.

YELLOW PIGMENTS AND THE FAT-SOLUBLE VITAMINE IN BEEF FATS

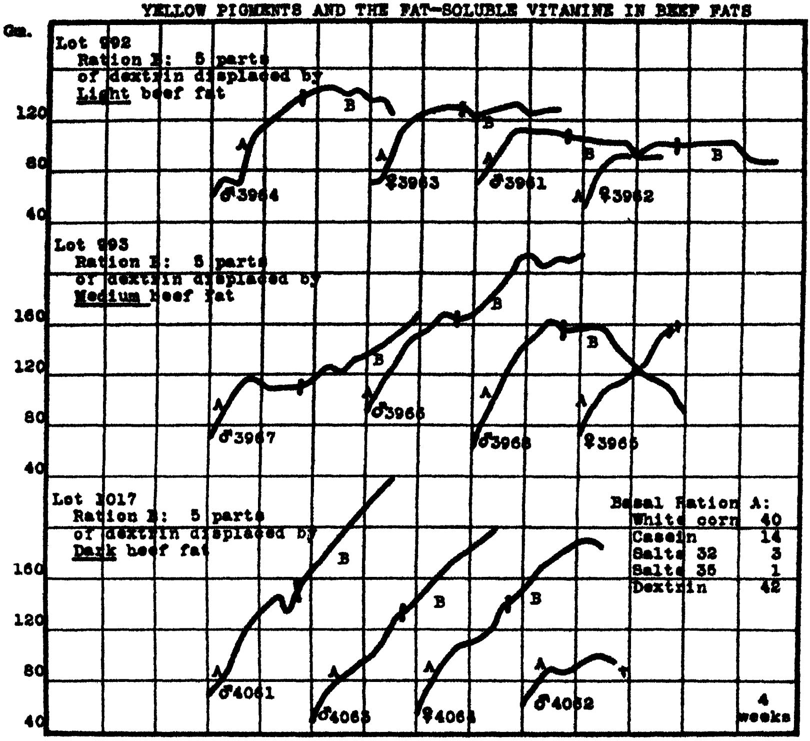

CHART 7. Chart 7 illustrates the relative efficiency of the same beef fats illustrated in Chart 6 as determined by their ability to induce recovery in rats which had given indisputable evidence of a fat-soluble vitamine deficiency in the ration.

In Lot 992, Rats 3961 and 3963 had edematous eyes, respectively, at the end of the 6th and 7th weeks on the white corn ration. In the case of the former, temporary improvement was noted from time to time; in the latter, permanent improvement extended over a period of 6 weeks, but in neither case was growth resumed. This illustrates, what we have often observed, that in the vast majority of cases less of the vitamine is required to maintain normal eye conditions than to maintain growth. In Rats 3962 and 3964 the eyes were inflamed severely at the time of discontinuation of the trial.

In Lot 993, on the medium beef fat, Rat 3967 indicated incipient inflammation with slight edema of the conjunctiva which was promptly cured upon change of ration. Rats 3966 and 3968 also showed some indications of an edema; in the former it persisted in spite of change of ration.

On the dark beef fat in Lot 1017 previous to the change all rats except Rat 4064 showed a severe ophthalmitis. Rat 4064 showed an enophthalmia. In all cases where the change of ration was made improvement was prompt and recovery complete.

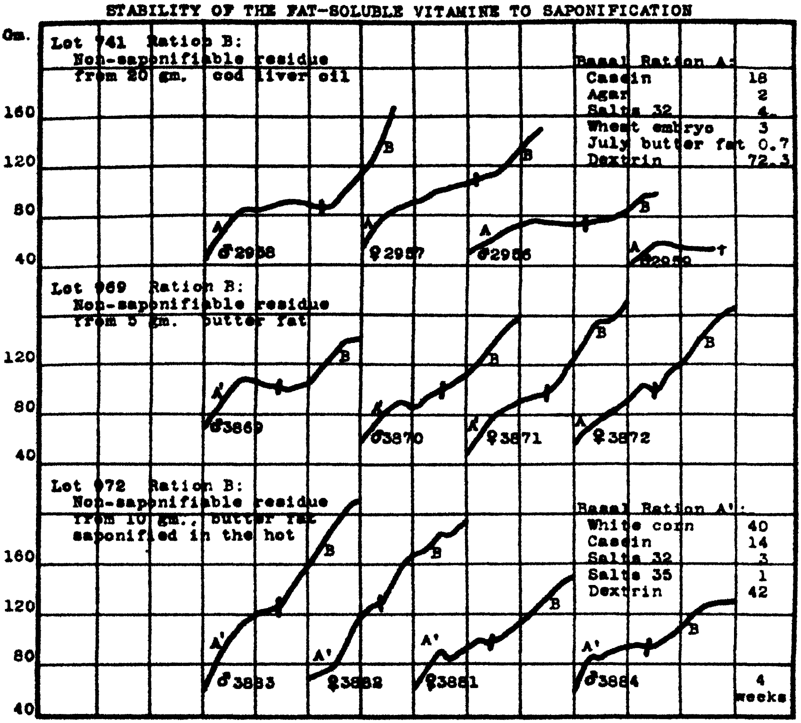

Chart 8. This chart shows the resistance of the fat-soluble vitamine to destruction by saponification of the fats in cod liver oil and butter fat as indicated by the prompt recovery of growth in the rats when an ether extract of a solution of the soaps was added to the basal rations. This consisted, in Lot 741, of a ration which had served in an experimental series to determine the fat-soluble vitamine content of butter fat. It is noteworthy that 0.7 per cent of this sample of July butter fat furnished no appreciable amounts of the vitamine. In Lots 969 and 972 our usual white corn ration was used. In no case, previous or subsequent to the change of ration, was any abnormality of eye conditions observed.

SUPPLEMENTARY PROTEIN VALUES IN FOODS.

I. THE NUTRITIVE PROPERTIES OF ANIMAL TISSUES.

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(Received for publication, March 21, 1921.)

In 1915 McCollum and Davis[1] described a systematic procedure for evaluating each of the several essential factors in foodstuffs. This procedure involves the feeding of the food under consideration as the sole source of nutriment to one group of animals, and to another the same food supplemented with single and multiple additions of purified foodstuffs in every possible combination. Such additions include protein, inorganic salts, a source of fat-soluble A, water-soluble B, and as was later pointed out by Chick and her coworkers,[2] Cohen and Mendel,[3] and others, water-soluble C. The latter factor is not essential in the diet of the rat. This procedure constitutes a biological method for the analysis of a foodstuff, and has been adopted by several students of nutrition. It has yielded results which have profoundly changed our basis of judgment as to the quality of a diet.

Studies from several laboratories have established the general landmarks which enable us to appreciate the lines of procedure which must be followed if satisfactory diets are to be made up by combining the various types of animal and vegetable foods. In order to make such combinations of foodstuffs it is necessary that we should understand in detail the special qualities of each of the important natural foods. Such an understanding can be secured only through carefully planned experiments on animals in which each food is studied as the sole source of nutriment, then studied in combination with each of the other foods with which it may be used in practice. It is necessary to proceed from the simple to the complex mixtures in these studies. Ultimately it is hoped that diets can be planned which will promote the optimum of physiological well being, and therefore lead to the optimum in physical development, length of life, and the preservation of youthful characteristics.

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[1] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

[2] Chick, H., and Hume, E. M., *Tr. Soc. Trop. Med. and Hyg.*, 1917, x, 141. Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

[3] Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

In publishing this series of papers dealing with the studies of the dietary properties of several types of food mixtures, several new observations will be pointed out. The interpretation of the results is based upon more careful and thorough observations than have hitherto been described in any similar studies. They include not only the rate and extent of growth, the fertility, and success in rearing of young, but also the period of life up to and including the onset of old age with its characteristic changes.

Since animal tissues have a very prominent place in the diet of man in most parts of the world, it is of great moment to understand the value of these with respect to each of the essential dietary factors. Our knowledge of the nutritive qualities of animal tissues is still very incomplete. Watson and Hunter[4] showed that rats fed exclusively on muscle meats suffered severe malnutrition. Liver has, however, found great favor as a food for young fish in hatcheries. It has been shown[5] that lard does not contain appreciable amounts of fat-soluble A, whereas fats extracted from a glandular organ (pig kidney or cod testicle) are a good source of it.[6] Liver and kidney have been shown[7] to be a good source of both fat-soluble A and water-soluble B, whereas muscle tissue is very poor in both.

Heart, a variety of muscle, was found on the other hand to contain sufficient of both fat-soluble A and water-soluble B to support growth for a time at least in young rats.[7]

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[4] Watson, C., and Hunter, A., *J. Physiol.*, 1906, xxxiv, 111.

[5] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641.

[7] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 179. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309; 1918, xxxiv, 17.

McCollum[6] pointed out that the mineral content of animal tissues such as muscle and glandular organs resembles that of seeds of plants sufficiently to indicate that it would not prove satisfactory as a source of inorganic elements in animal nutrition.

No data exist showing the comparative values of the proteins of the several kinds of animal tissues. Osborne and Mendel[7] have shown that growth takes place in young rats restricted to 18 per cent of protein derived solely from liver, kidney, muscle, or brain. This is a liberal amount, and is far above the plane of intake necessary for the support of normal growth in the rat when the quality of the protein is good.

There is also reason to inquire into the possibility of the presence of toxic substances in the glandular organs. The presence of such powerful physiological stimulants as thyroxin and adrenalin in the thyroid and suprarenal glands, respectively, makes them unfit for human or animal food. We have been informed that the Eskimos do not eat the liver of the reindeer. This fact may perhaps be accounted for by the absence of a gall bladder in this species, and consequent high content of bile, which would give it a bitter flavor. The Eskimos are said not to eat the liver of the polar bear, to avoid that of a certain species of seal, and to believe the liver of the dog poisonous. The liver is concerned with so many types of transformations of organic substances which have their origin in metabolism that it seemed possible that certain of these may be present in sufficient amounts to be detrimental to one who eats freely of it. Similar consideration might lead one to inquire whether the kidney of an animal may contain sufficient amounts of certain metabolic products as to render it undesirable as a food.

The glandular organs are rich in cell nuclei and consequently yield considerable amounts of purines when metabolized. These ultimately are converted in great measure into uric acid. There are conditions of perverted metabolism in man in which the excretion of uric acid and urates is interfered with and doubtless under such circumstances liver or kidney should not be eaten. There would seem little reason, however, why these organs should not be eaten by healthy persons as adjuvants to the diet. They possess dietary properties as distinct from those of muscle tissue as the leaves of plants do in contrast to the seeds.

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[6] McCollum, E. V., The newer knowledge of nutrition, New York, 1918.

In regions such as Labrador and Newfoundland where the diet of the habitants consists essentially of wheat flour, molasses, fish, meats, tea, and raisins, beri-beri and scurvy are common. A condition popularly called night-blindness is also of frequent occurrence among these people. On such a diet one would suspect the danger of developing xerophthalmia and apparently this is the case. The successful treatment of night-blindness has recently been reported by the administration of cod liver oil. This oil is a good source of fat-soluble A and is very effective in the cure of xerophthalmia.

Fresh liver is rich in fat soluble-A, water-soluble B, and water-soluble C, the protective dietary factors for ophthalmai, beri-beri, and scurvy, respectively. It should be easily possible to eradicate these dietary diseases in such regions as Labrador and Newfoundland by the use of fish livers as food. It is strange indeed that the natives of these regions have failed to discover the value of a by-product of their fishing industry which would serve in a great measure to correct the conspicuous faults in their diet.

For the purpose of comparing the biological values of the proteins of kidney, liver, and muscle, we have followed the procedure described by McCollum, Simmonds, and Parsons[9] of feeding the tissues singly as the sole sources of protein at planes so as to introduce 9 per cent of protein into the food mixture. They were supplemented with respect to all other factors so as to make a satisfactory diet, with the possible exception of the protein moiety. Lots 2475, 2476, and 2474, Chart 1, represent experiments of this type. Such experiments make it possible to compare the proteins of these animal tissues with those of a number of combinations of cereal and other seed proteins with milk which we have previously studied.[10] It has been shown that normal growth is secured when the diet contains 9 per cent of protein of high biological value. If much less than this content of protein is furnished by the food mixture, even when the protein is excellent, the growth falls distinctly below the curve of normal expectation.

[9] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

[10] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 415.

When proteins of several types such as those of cereals, legume seeds, and milk were compared in this manner it was found that the proteins of milk were superior to those of most seeds of plants yet examined. The seed which furnishes proteins nearest to milk in value for conversion into body proteins during growth is wheat, which therefore stands first among the cereals in value. From the records of Chart 1, which show the effects of feeding kidney, liver, and muscle proteins respectively at 9 per cent of the food mixture, it is apparent that the proteins of these substances are scarcely superior to those of certain cereals, especially wheat. We base this conclusion on the growth of young rats restricted to one of these sources of protein at the critical 9 per cent level. Normal growth is not secured with wheat proteins fed below this plane of intake.

This result is very surprising indeed. It seems best explainable on the assumption that the patterns on which the proteins of the muscle are constructed differ very decidedly from that of the liver or kidney, and presumably from the glandular organs as a class. The nutritive needs of the body involve the replacement of tissues of both organ and muscle types. Apparently glandular organs or muscle as the sole source of nitrogen in the food fails to serve for efficient transformation into new body proteins during the symmetrical growth of the body tissues. They are complete but their transformation into body proteins cannot be very effectively accomplished (Chart 1).

We have shown in a previous communication that the cereal grain proteins do not in general make good each other's deficiencies or enhance to any great extent each other's biological values when two of them are combined.[9] Rye and flaxseed meal proteins form a notable exception. These, when combined in certain proportions, form a mixture which is distinctly superior to the proteins of either component alone. The supplementary value of one protein for another depends on the yields by each of those indispensable amino-acids which are present in each of the sources in smallest amounts. We shall show in one of the following papers that kidney, liver, and muscle proteins have much greater values as supplements to the cereal proteins than the cereal or legume proteins have, with few exceptions, among themselves. The data in the succeeding paper illustrate the importance of animal tissues in the food supply when the diet consists mainly of such vegetable products as do not yield a mixture of proteins having a high biological value. For the special purpose of enhancing the quality of the protein in the diet they have the highest value. It must be kept in mind, however, that their use does not correct the mineral deficiencies of a cereal, tuber, and legume seed diet, and that when muscle meats are used as food they have little effect in raising the content of fat-soluble A in the resulting mixtures. In no instance, therefore, will one of these types of animal tissue supplement a cereal, tuber, and fleshy root type of diet so as to make it highly satisfactory.

Our observations on the rats described in these experiments do not show any definite evidence of injury to the animals as the result of being fed excessively high protein diets. These diets were, however, essentially complete and fairly well proportioned as regards all factors other than protein. It is not justifiable to generalize from these results that such a high protein intake is safe for man. Our animals were not kept to determine the possible span of life or the time of appearance of the signs of senility, owing to the necessity of temporarily vacating the room in which the animals were kept. It is the custom for people in the United States to derive a high protein diet, when such is taken, in great measure from muscle meats, fish, poultry, eggs, and legume seeds. Menus containing such high protein foods will only in exceptional cases be completely supplemented by other constituents of the diet. The evil effects often attributed to excessive protein consumption may now with some confidence be attributed in many instances to faults in the composition of the diet in factors other than protein. Further studies are required to demonstrate the relative merits of diets of high and low protein contents when other factors are of comparable value in the two cases. We shall discuss this phase of nutrition on the basis of carefully planned experiments on animals in a later communication.

There are some very interesting and important instances of successful nutrition among people who have lived almost exclusively upon a diet very rich in protein and derived from foods of animal origin. We are indebted to Mr. Vilhjalmur Stefansson for the information that previous to about 1850 dental caries were very rare or absent from Iceland. During the last half of the 19th century cases have gradually become more and more common until today infected teeth are perhaps as common there as in most parts of the United States. No carious teeth were found among 96 skulls disinterred by Stefansson from a cemetery in Iceland dating from the 9th to the 13th centuries. These skulls are now in the Peabody museum at Harvard University and have been described by Hooton.[11] The diet of the Icelanders previous to about 1850 consisted essentially of milk, mutton, fish, and fowl, but in some parts of the island they ate the eggs of wild birds. The only vegetable food eaten regularly was carrageen moss, but potatoes and turnips were eaten to some extent. The teeth of the natives and their general health were excellent as long as this diet was taken. The deterioration of the teeth apparently began about the time when cereals and sugar were regularly imported into Iceland as sources of food.

The teeth of the primitive Eskimo were excellent. The younger generation in northern Alaska, whose diet is derived in a large measure from cereal grain products, canned foods, and muscle meats, similar to what would be purchased in a grocery store in the United States, has poorly calcified teeth which are often carious.

We have collected numerous observations on the effect of dietary faults on the quality of the skeleton in the rat. These data make it clear that most profound differences in the extent of calcification and density of the deposited calcium phosphate can be effected by such faults as are found in the cereal, tuber, and muscle meat type of diet.

The diet of the primitive Eskimo was very rich in protein but it was at least fairly satisfactory with respect to other factors. It consisted of muscle tissue and fat as the principal components, but all blood was carefully saved and eaten, and the glandular structures were regarded as dainties of especial delicacy. In addition, they regularly ate bone marrow and chewed the softer parts of bones, such as ribs and the epiphyses of the long bones. Such a selection of tissues suffices for the satisfactory nutrition of the rat and produces the fine physical development seen in the Carnivora such as the lion, tiger, jaguar, etc. The deterioration of the teeth of the Eskimo which occurred simultaneously with the modification of the diet due to contact with the white man is in harmony with what we have been led to expect as the result of experimental studies of the types of combinations of ordinary foods which enter into the diet of man and animals in different parts of the world. The cereal and muscle meat diet or its equivalent, the bread, meat, and potato type of diet, is in all probability the cause of the deterioration of the teeth of the present generation of "civilized" Eskimos as it is among the people of the United States and Europe.

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[11] Hooton, E. A., *Am. J. Physical Anthropol.*, 1918, 1, 53.

CONCLUSIONS.

The kidney, liver, and muscle of the ox contain proteins which, when they serve as the sole source of nitrogen, and are fed singly as the sole source of protein, but completely supplemented with respect to all necessary factors other than protein, are shown to possess about the same biological value as those of the wheat kernel.

There is no distinct evidence of toxicity in either muscle, kidney, or liver tissue when fed at planes of intake sufficiently high to introduce from 35 to 70 per cent of protein into the diet.

The first limiting factor in the kidney, liver, and muscle tissue is a lack of calcium. It is also necessary to add sodium chloride in order to insure prolonged well being. Carnivorous man and animals secure their sodium chloride by eating blood, and calcium by eating bone. Liver and kidney contain an abundance of fat-soluble A and of water-soluble B, and when fresh and raw, of water-soluble C. Muscle tissue is very deficient in these factors but does not entirely lack any one of them. Kidney proteins appear to have higher biological value than those of the other animal tissues yet studied.

It has been our custom for years in preparing experimental diets to thoroughly grind the several components of the food and to make a uniform mixture from which the constituent parts cannot be picked out by the animals. In all cases iodine was given once a week in the form of potassium iodide-iodine in the drinking water, which was distilled. The liver, kidney, and steak, except when the contrary is stated, were steamed in a sterilizer until thoroughly cooked, subsequently dried, and ground. Practically all visible fat was removed from both organs and muscle. The curves presented in the charts are typical representatives of a group of four to six animals which composed each experimental group.

Chart 1.—The curves in this chart illustrate the growth of young rats fed diets containing 9 per cent of protein derived solely from beef kidney, liver, and muscle, respectively. The inorganic additions were of a character which completed, at least in a fairly satisfactory manner, the mineral content of these animal tissues. There can be no doubt that kidney proteins have a somewhat higher biological value than those of liver. Liver proteins, surprising as it may seem, have not been found in our experiments to be superior to those of muscle (steak).

In Lot 2475 there were two females. One of these died in parturition. The other at the age of 4 months had one litter of four young and successfully reared them. She died from unknown cause about 40 days after weaning her young. Two of her daughters grew up and produced one and three litters of young (a total of seventeen), respectively. Of these ten were successfully weaned.

Two granddaughters of the female described in the original experimental group were kept 6½ months on the family ration, and although they appeared to be in good condition, neither proved fertile. The rats fed the kidney ration did not exhibit early signs of aging. In this respect they were superior to the groups fed diets containing comparable amounts of liver and of muscle.

The records of these experimental groups fed 9 per cent of protein from kidney, liver, and muscle, respectively, all other factors being more or less satisfactorily adjusted, show them to be typical examples of nutritional instability. In Lot 2475 on the kidney diet, each succeeding generation was inferior to its parents. Lack of uniformity of vitality among individuals of the same group or family is observed with striking frequency in animals whose diets fall but little below the quality necessary to maintain the vigor of the species unimpaired throughout successive generations.

It might be suggested that the failure of the animals fed liver to develop more satisfactorily, was due to the presence of toxic substances in this organ, which performs the function of degrading numerous foreign and poisonous substances derived from metabolism and absorption from the alimentary tract and elsewhere. The records of Chart 2 show clearly that this is not the case.

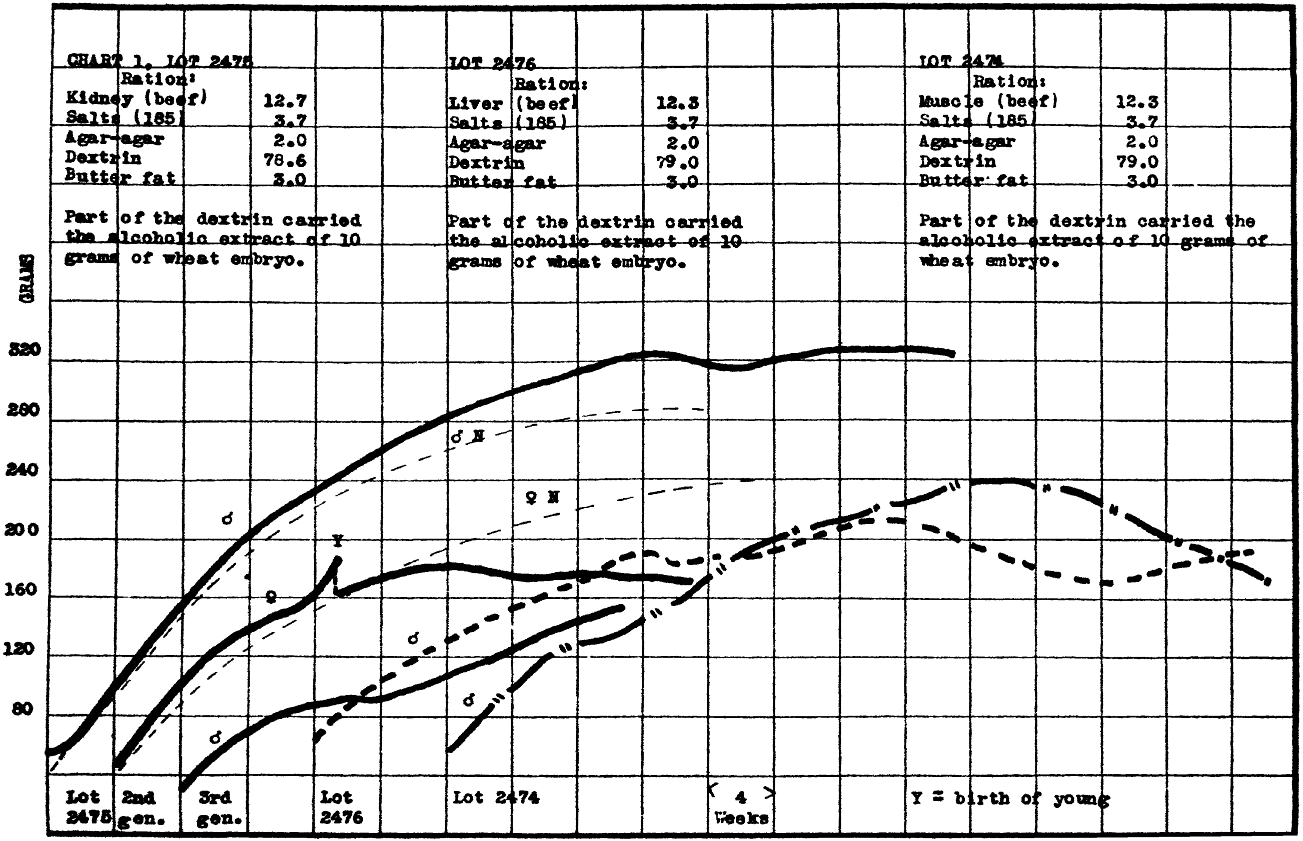

CHART 1. LOT 2475

Ration:

Kidney (beef)	12.7
Salts (185)	3.7
Agar-agar	2.0
Dextrin	78.6
Butter fat	3.0

Part of the dextrin carried the alcoholic extract of 10 grams of wheat embryo.

LOT 2476

Ration:

Liver (beef)	12.3
Salts (185)	3.7
Agar-agar	2.0
Dextrin	79.0
Butter fat	3.0

Part of the dextrin carried the alcoholic extract of 10 grams of wheat embryo.

LOT 2474

Ration:

Muscle (beef)	12.3
Salts (185)	3.7
Agar-agar	2.0
Dextrin	79.0
Butter fat	3.0

Part of the dextrin carried the alcoholic extract of 10 grams of wheat embryo.

Y = birth of young

Neither of the two females fed the steak diet had any young, although they appeared to be well nourished and were kept under observation during more than 12 months.

Three females on the liver diet were somewhat undersized, aged early, and never had any young, although they were kept under observation for more than a year. In the case of both the liver and muscle diets this was apparently due to the proteins from these sources not being of sufficiently good quality to make 9 per cent of protein from these sources sufficient to promote well being at the optimum, since in these diets all other factors were corrected.

Composition of Salt Mixture 185.

	per cent
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4 + H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2$	0.540
Fe citrate	0.118
Ca lactate	1.300

Chart 2.—The rats in these experiments were fed food mixtures which were satisfactorily constituted except for possible shortage of water-soluble B and fat-soluble A, which in each case were derived entirely from 25 per cent of kidney, liver, or muscle, respectively. The growth curves and fertility of the animals on the kidney and liver diets show that these amounts were satisfactory as sources of fat-soluble A and water-soluble B. In these diets the protein of the animal tissues was supplemented with 9 per cent of casein. Chart 4 shows, however, that 20 per cent of either kidney or liver suffices as the sole source of protein for normal growth, reproduction, and rearing of young, for the fourth generation on the kidney diet appeared to have normal vitality. The rats of the fourth generation on the liver diet were inferior, although they were successfully weaned.

Two females on the kidney diet, Lot 2163, had collectively four litters (fourteen young), all of which were reared. Two of the daughters were maintained during 10 months on the diet on which their mothers had lived. One remained sterile, the other had a single litter (five young) at about 4 months of age and never became pregnant afterwards. The young were in good condition when weaned.

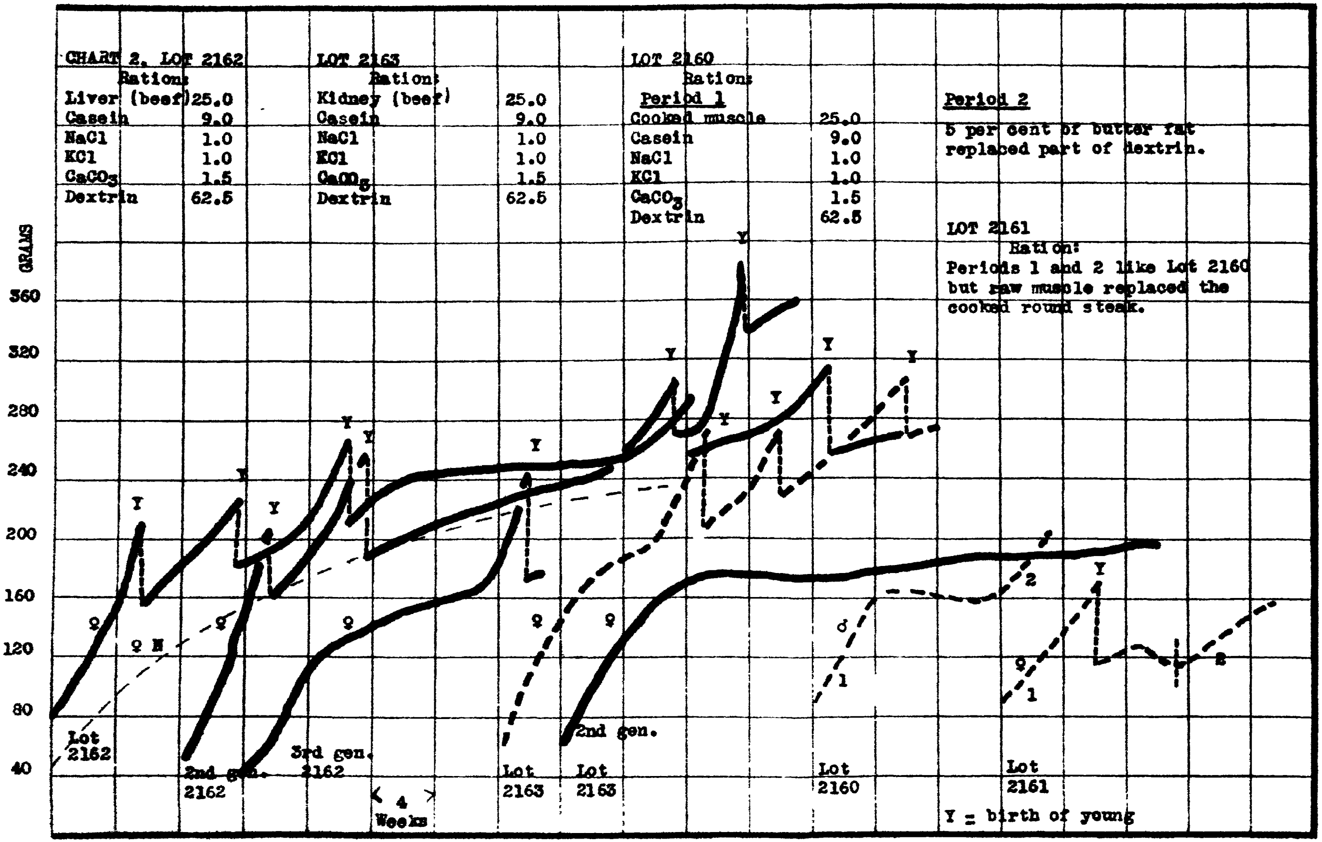
CHART 2. LOT 2162
Ration:
Liver (beef) 25.0
Casein 9.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 62.5
LOT 2163
Ration:
Kidney (beef) 25.0
Casein 9.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 62.5
LOT 2160
Ration:
Period 1
Cooked muscle 25.0
Casein 9.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 62.5
Period 2
5 per cent of butter fat replaced part of dextrin.
LOT 2161
Ration:
Periods 1 and 2 like Lot 2160 but raw muscle replaced the cooked round steak.
GRAMS
360
320
280
240
200
160
120
80
40
Y
♀
♂
N
1
2
Lot 2162
2nd gen. 2162
3rd gen. 2162
< 4 > Weeks
Lot 2163
Lot 2163
2nd gen.
Lot 2160
Lot 2161
Y = birth of young

There were three females raised on the liver diet (Lot 2162). They had collectively fifty-six young (nine litters), of which thirty-three were weaned. The young which died became lethargic just before weaning time and died in this condition. We have at present no explanation for this peculiar behavior.

Lots 2160 and 2161 were fed diets comparable to Lots 2162 and 2163, except that the factors, fat-soluble A and water-soluble B, were entirely derived from 25 per cent of muscle tissue (cooked and raw beefsteak, respectively). In both cases there was failure of growth after about 4 weeks. After a period of suspended growth during which one female in Lot 2161 had a litter of four young which were eaten by the mother shortly after birth. 5 per cent of butter fat was added to the diet of each group. In both cases there was a marked response to growth following this addition. This demonstrates that 25 per cent of round steak does not contain sufficient fat-soluble A to meet the needs of the young rat. Lot 2142, Chart 3, shows clearly that 20 per cent of steak does not furnish sufficient water-soluble B for the normal nutrition of young rats during growth.

The records in this chart show clearly the remarkable difference between the glandular organs as compared with muscle tissue in respect to their content of both the factors, fat-soluble A and water-soluble B. This supports the view which we have repeatedly stated, that the dietary properties of a substance can be fairly accurately predicted from a knowledge of their biological function.

Chart 3.—The three groups of animals whose curves are shown in this chart were fed diets comparable in all respects, except that the sole source of water-soluble B and protein was 20 per cent of the dry matter in the diet in the form of kidney, liver, and muscle, respectively. A comparison of these curves with those of Chart 1 shows that the protein furnished by this proportion of liver, kidney, and steak, respectively, suffices to support normal growth when other factors in the diet are properly adjusted.

The animals fed the kidney ration, Lot 2144, were markedly superior to the other two groups. Representatives of four successive generations were grown upon this diet as their sole source of water-soluble B, and with no evidence of deterioration. In the original group there were three females. These produced collectively ten litters (forty young) but only six individuals were

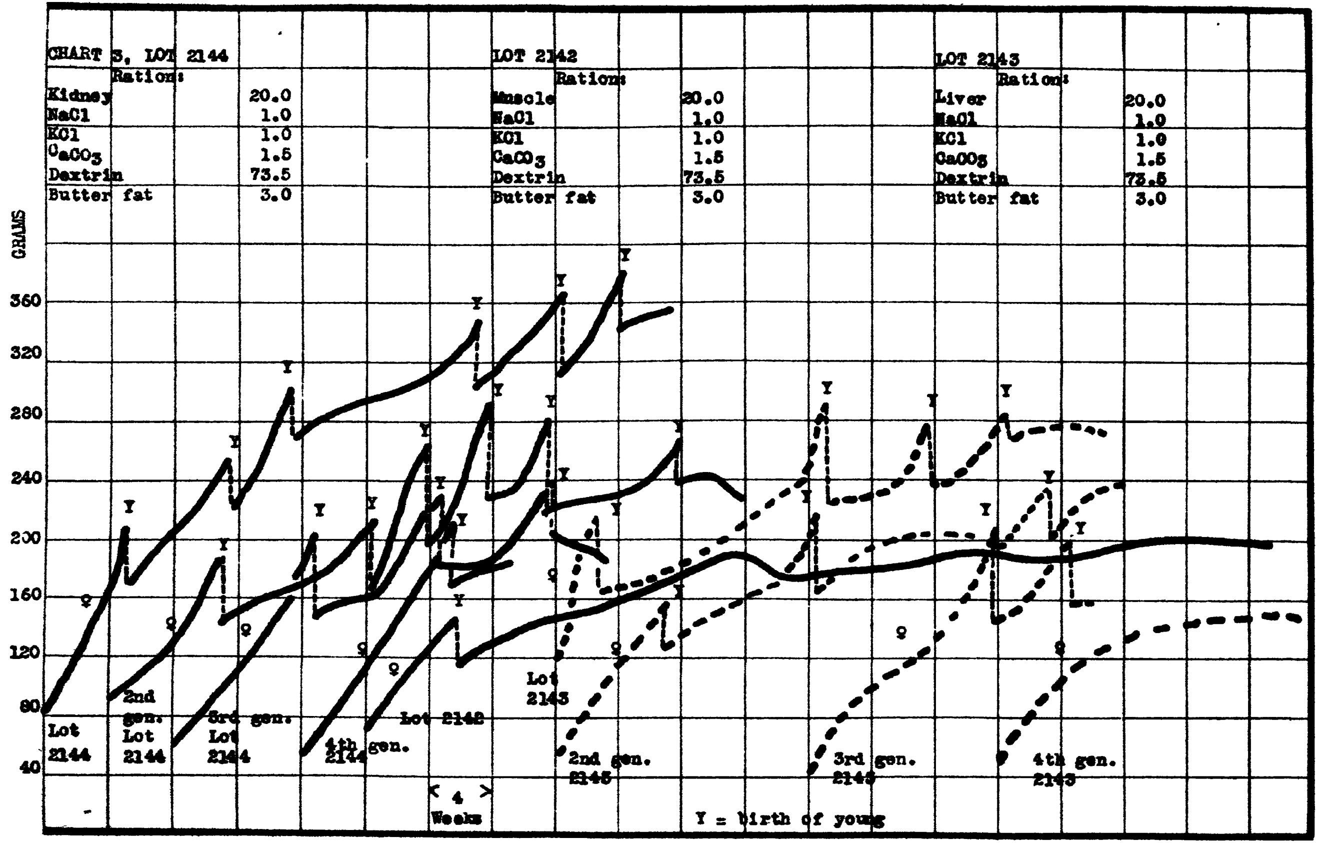
CHART 3, LOT 2144
Ration:
Kidney 20.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 73.5
Butter fat 3.0
LOT 2142
Ration:
Muscle 20.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 73.5
Butter fat 3.0
LOT 2143
Ration:
Liver 20.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 73.5
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
Lot 2144
2nd gen. Lot 2144
3rd gen. Lot 2144
4th gen. 2144
Lot 2142
Lot 2143
2nd gen. 2143
3rd gen. 2143
4th gen. 2143
♀
Y
< 4 > Weeks
Y = birth of young

successfully weaned. The mothers destroyed their young soon after birth.

Two daughters of the females just described were kept on the family diet. They had together six litters (forty young) and weaned only four individuals altogether. Here again the mortality was the result of cannibalistic tendencies in the mothers. But one granddaughter of the original group was kept on the family diet. She grew normally and had six young (two litters) and successfully weaned all of them. One great granddaughter was brought up on the diet. She had three young (one litter) and weaned them successfully. With optimum nutrition these litters would have been two or three times as large as those produced.

It might be suggested that perhaps the content of water-soluble B in this diet was below the optimum, and that for this reason the nutrition of the nervous systems of the rats restricted to this diet was faulty, and that the tendency to destroy the young was an expression of an abnormal psychology analogous to the psychosis observed in beri-beri or pellagra. The fact that the third and fourth generation females in this family were less cannibalistic than their mothers and grandmothers militates somewhat against this view.

Lot 2143 derived all its antineuritic factor and protein from 20 per cent of liver in the diet. This was equivalent to 14.4 per cent of protein. The growth curves were normal. In the first group fed this diet there were two females. These had forty-six young (eight litters) and of these but twenty were weaned. Two second generation females had eighteen young (four litters) and weaned ten of them. Two third generation females had thirteen young (two litters) of which they weaned six. One fourth generation female was kept 5½ months, when she died, never having had any young. None of these animals in any generation was kept to an advanced age. The young of the rats fed the liver diet were somewhat undersized but appeared to be vigorous.

Lot 2142 was fed a diet comparable to the others described in this chart, but with all protein and water-soluble B derived from 20 per cent of muscle (round steak). These animals were markedly inferior both as regards growth and fertility to those fed kidney or liver at the same plane of intake. Two females were kept to the age of 15 months, an age which usually marks the end of fertility in the rat. One had a single litter of eight young but they died when about 2 weeks old. The other remained sterile. The rats in this group were still in good nutritive condition after a year on the diet. This chart, like the other records in this paper, shows clearly the superiority of glandular organs over muscle tissue as sources of water-soluble B.

Chart 4.—These records show the behavior of young rats fed cooked dry beef kidney as the sole food, Lot 1253. Lot 1254 shows the growth curves of rats fed 97.5 per cent of kidney supplemented with sodium chloride and calcium carbonate. Lot 1255 was fed kidney, 94.5 per cent, supplemented with sodium chloride, calcium carbonate, and butter fat. Lot 1256 was fed a diet like that of Lot 1255 except that 14 per cent of the kidney was replaced by lactose. This last ration was designed to show whether lactose would tend to modify the bacterial flora of the alimentary tract. No beneficial effects of the modification were apparent.

The rats restricted to cooked dried kidney as their sole food grew in a fairly normal manner. Two females had collectively fourteen young (a litter each), five of which were weaned. One of these young developed abnormal ribs suggestive of rickets. None of these young grew up.

It is very remarkable that young rats confined to a diet of kidney could develop so successfully and reproduce and rear young. This tissue is very poor in calcium, and yields a great excess of acid on being metabolized. The protein content of this diet was not far from 71 per cent, yet because all essential food factors except calcium were so abundant, the animals were able to tolerate this deficiency and the abnormal protein content remarkably well.

Lot 1254 contained two females, one of which became pregnant and died in parturition. The other had a tumor which became so large that she was chloroformed.

There was but one female in Lot 1255. She had sixteen young (two litters) of which nine were weaned. Two second generation females had collectively seventeen young (a litter each) of which ten were weaned. Two third generation females had each a litter of young (seventeen) of which twelve individuals were weaned. The young appeared to be well developed, but were always greasy from their food. They drank much water. The cage had a strong odor. The protein content of the ration of Lot 1254 was 69 per

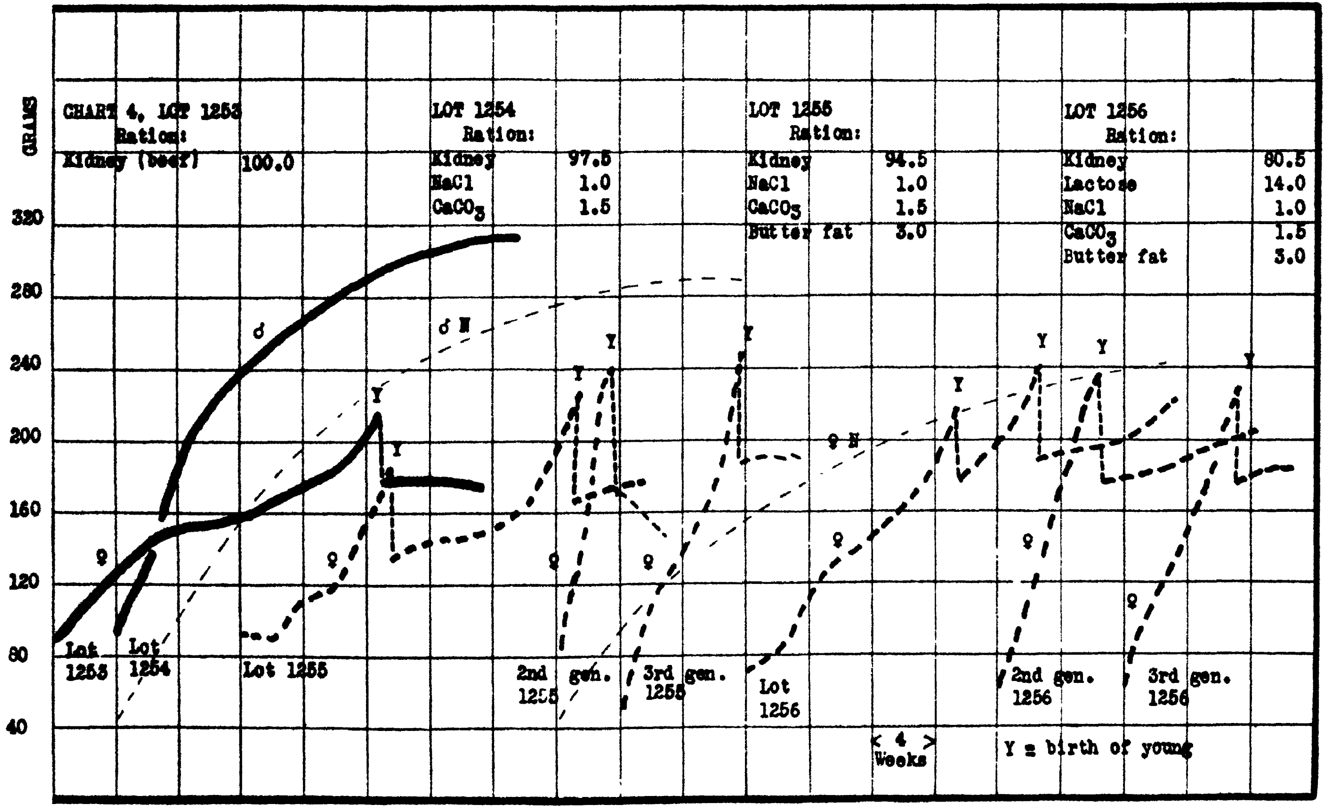
CHART 4, LOT 1253
Ration:
Kidney (beef) 100.0
LOT 1254
Ration:
Kidney 97.5
NaCl 1.0
CaCO3 1.5
LOT 1255
Ration:
Kidney 94.5
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
LOT 1256
Ration:
Kidney 80.5
Lactose 14.0
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
♂
♀
♂ N
♀ N
Y
Lot 1253
Lot 1254
Lot 1255
2nd gen. 1255
3rd gen. 1255
Lot 1256
2nd gen. 1256
3rd gen. 1256
< 4 > Weeks
Y = birth of young

cent; that of Lot 1255, 67 per cent; of Lot 1256, 57 per cent. The results show that the rat is capable of growing and remaining in a state of health on a diet comparable to that of the strictly carnivorous animals. At least among the carnivorous animals of the Arctic regions the proportion of protein in the diet is not necessarily excessive at all times. Mr. Stefansson has informed us that it is not unusual for travelers to come upon a seal that has been killed and skinned, the subcutaneous layer of fat eaten, and the remainder of the carcass left practically untouched. The polar bear evidently prefers fat to protein as a source of energy.

Lot 1256 contained two females. They had collectively twenty-two young (two litters each) of which fifteen were weaned; two second generation females had together nineteen young, and weaned fourteen of them. One third generation female had one litter of six young and weaned them all.

Chart 5.—The diet of the four groups of rats whose curves are shown in this chart was in all instances comparable to those in Chart 4, the only difference being the substitution of liver for kidney in the diets of Lots 1277, 1278, 1279, and 1280.

Lot 1277, which was confined to liver as its sole food, grew very little and died from 4 to 6 months after being confined to the diet.

Lot 1278 was fed liver supplemented with sodium chloride and calcium carbonate. On this diet growth was approximately normal. The group contained three females. One of these had twelve young (two litters) and weaned eleven of them. The other two remained sterile. One second generation female remained sterile.

Lot 1279 was fed liver supplemented with sodium chloride, calcium carbonate, and butter fat. Lot 2162, Chart 2, shows that even 25 per cent of liver as the sole source of fat-soluble A furnishes a sufficient amount of this factor.

There were three females in this group. They had thirty-four young (six litters) and weaned thirty-one of them. One second generation female grew up on the diet, and had six young in one litter. She weaned four of these. One third generation female in this family had a litter of six and weaned them all. The reproduction records of this group were distinctly better than those of Lot 1278, whose diet was identical except for the 3 per cent of but-

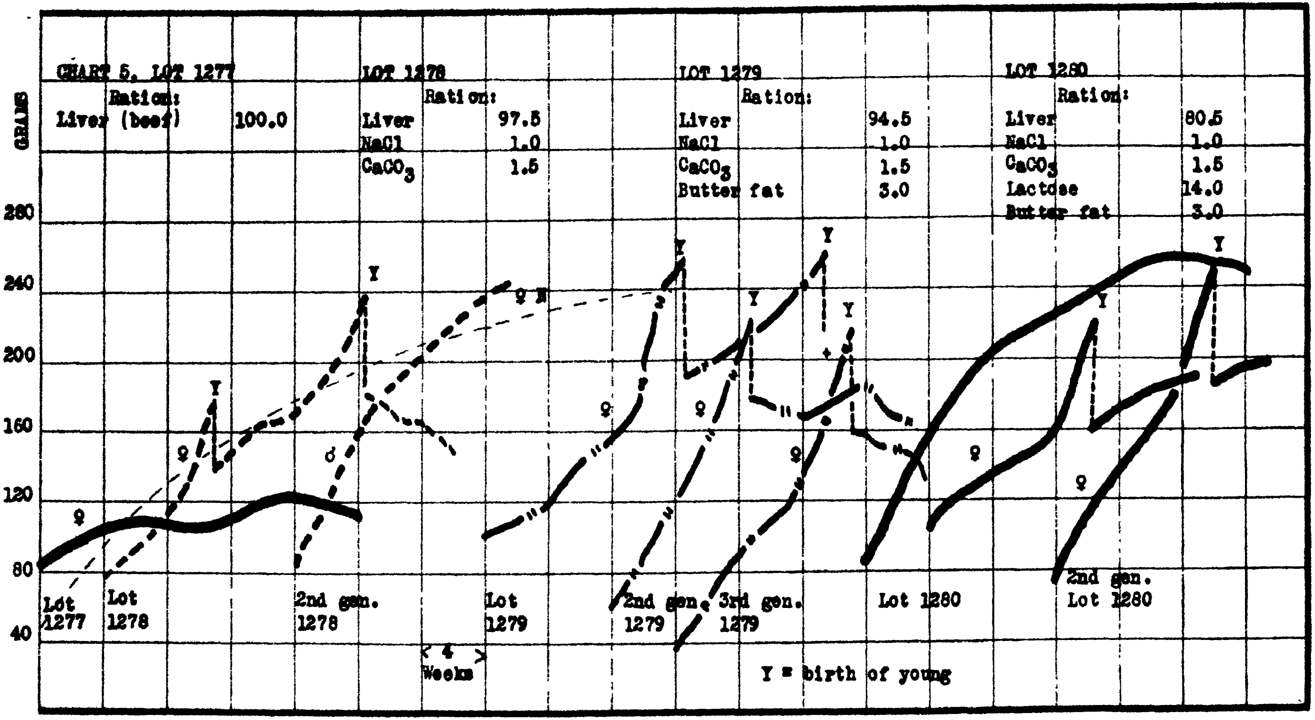
CHART 5. LOT 1277
Ration:
Liver (beef) 100.0
LOT 1278
Ration:
Liver 97.5
NaCl 1.0
CaCO$_3$ 1.5
LOT 1279
Ration:
Liver 94.5
NaCl 1.0
CaCO$_3$ 1.5
Butter fat 3.0
LOT 1280
Ration:
Liver 80.5
NaCl 1.0
CaCO$_3$ 1.5
Lactose 14.0
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
Lot 1277
Lot 1278
2nd gen. 1278
Lot 1279
2nd gen. 1279
3rd gen. 1279
Lot 1280
2nd gen. Lot 1280
< 4 > Weeks
Y = birth of young
Y
♀
♂

ter fat. We cannot explain the reason for the superiority of Lot 1279.

Lot 1280 was like Lot 1279 except that 14 per cent of lactose replaced a like amount of liver. This did not exert any noticeably favorable effect on the well being of the animals. Two females each had a litter (collectively fourteen young) and weaned thirteen individuals. One second generation female had one litter of eight young and weaned seven of these. The young in Lots 1279 and 1280 appeared to be strong and in good condition but showed in all cases a peculiar condition which we have observed occasionally in abnormal animals; *viz.*, a wet and stained area around the urethral orifice. This not infrequently occurs in poorly nourished animals.

Chart 6.—The animals whose records are shown in this chart were fed a diet made adequate in all respects as far as could be judged, and with the protein derived solely from 50 per cent of dry beef liver. The protein content of the diet was about 35 per cent.

Lot 1281 contained three females. These had collectively thirty-five young (five litters) and weaned twenty-two. Two second generation females had one litter each, collectively nineteen young, of which number they weaned seventeen. These young appeared to be very well nourished.

Lot 1282 contained two females. These collectively had five litters (thirty-four young). They weaned thirty-one of these young.

Two second generation females were restricted to this diet. One had a litter of six, which she successfully weaned. The other female remained sterile. The young were apparently well nourished but badly urine-stained. There was no evidence that the inclusion of lactose benefited the animals.

Chart 7.—The animals whose records are shown in this chart were fed either muscle tissue or blood, with and without certain purified food additions. Lot 1232 was restricted to a diet of cooked, dried, beef muscle. They were able to grow very slowly, but remained very much undersized, and died early. None of this group had any young. Two of the rats showed, toward the end of their lives, distinct signs of xerophthalmia, due to the lack of fat-soluble A in muscle tissue.

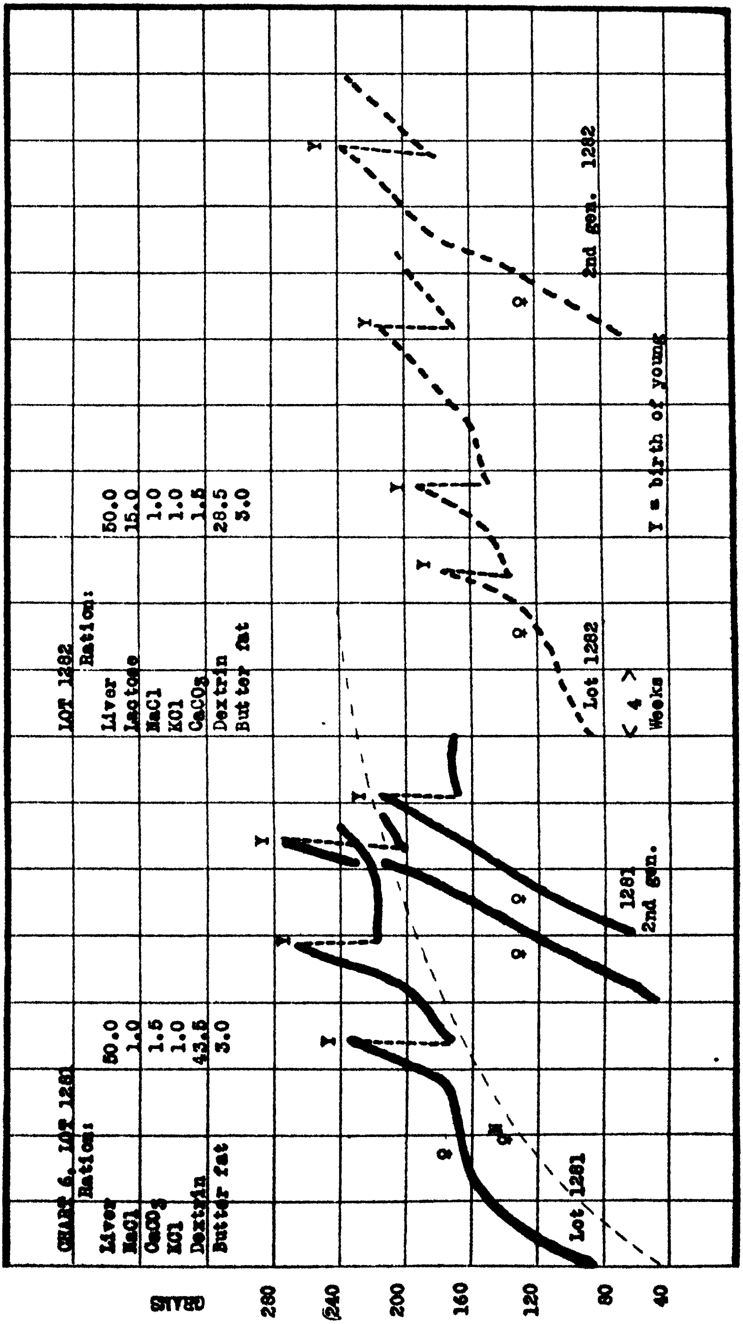

CHART 6. LOT 1281

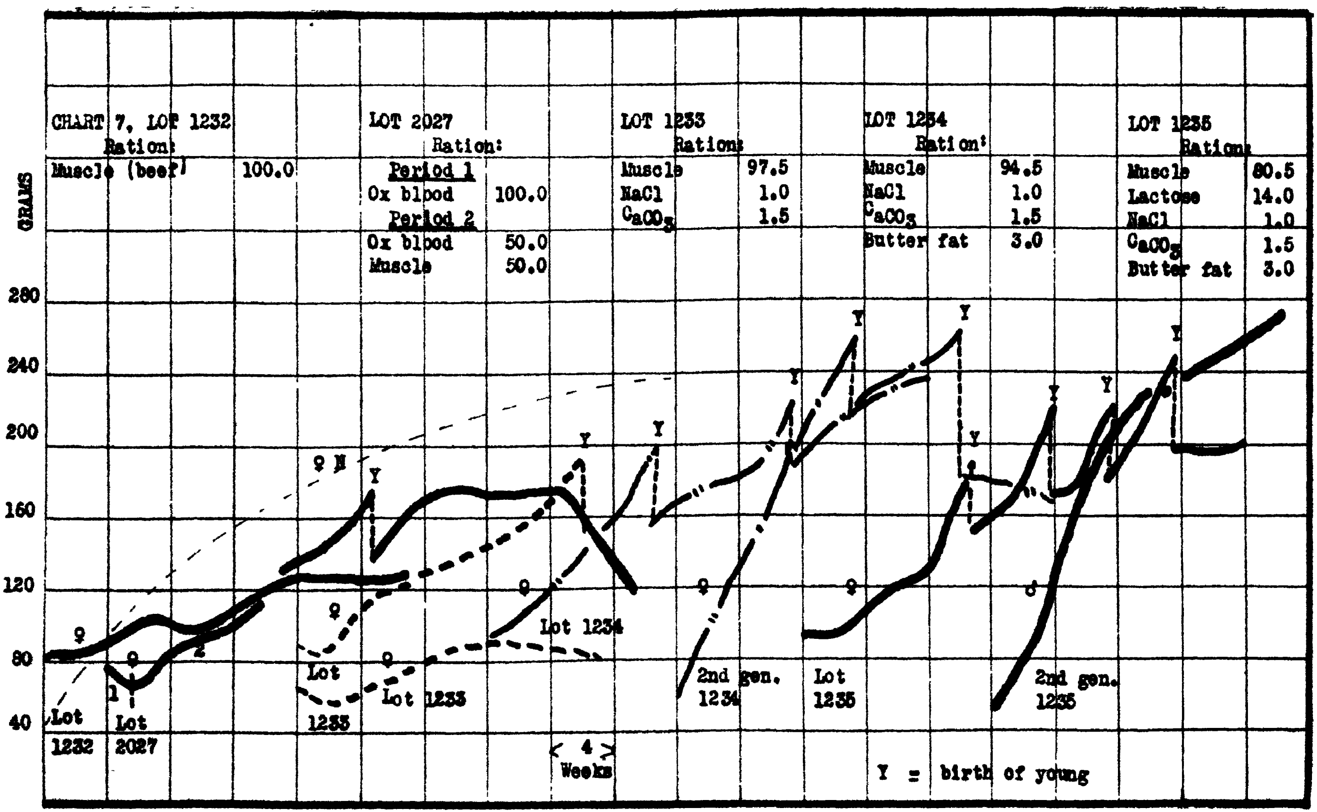
CHART 7, LOT 1232
Ration:
Muscle (beef) 100.0
LOT 2027
Ration:
Period 1
Ox blood 100.0
Period 2
Ox blood 50.0
Muscle 50.0
LOT 1233
Ration:
Muscle 97.5
NaCl 1.0
CaCO3 1.5
LOT 1234
Ration:
Muscle 94.5
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
LOT 1235
Ration:
Muscle 80.5
Lactose 14.0
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
Lot 1232
Lot 2027
Lot 1233
Lot 1233
Lot 1234
2nd gen. 1234
Lot 1235
2nd gen. 1235
< 4 > Weeks
Y = birth of young
♀ M
Y

Lot 2027 was fed dried ox blood as their sole food in Period 1. They declined rapidly on this and after 2 weeks were changed to a diet of muscle meat 50 per cent and dried blood 50 per cent. On this diet they were able to grow at a rate approximately half the normal. A female in this group had a single litter of four young, which she destroyed within a few days. All these animals showed early signs of old age.

Lot 1233 was fed cooked dried muscle (beefsteak) supplemented with sodium chloride and calcium carbonate. They grew but little better on this diet than on muscle alone, but one female had a litter of three young, which she destroyed soon after birth. Two other females remained sterile on this diet. That fat-soluble A was the limiting factor in this diet is shown by the records of Lots 1234 and 1235.

Lot 1234 was fed muscle, sodium chloride, calcium carbonate, and butter fat. They not only grew in an approximately normal manner, but were fairly fertile and had moderate success in the rearing of their young. Two females had together twenty young (three litters) of which sixteen were weaned. One second generation female was kept on the diet. She had one litter of five young and weaned them all. The young were somewhat inferior. Further studies are necessary to determine what modifications of this diet are necessary to secure greater fertility and higher vitality in the young.

Lot 1235 was fed a diet of beefsteak supplemented with sodium chloride, calcium carbonate, butter fat, and 14 per cent of lactose. They were not superior in vigor, fertility, or success in rearing young to Lot 1234 which had more beefsteak in place of the lactose. Two females had thirty-two young collectively (five litters) of which twelve were weaned. Since these were all males no further reproduction records were secured.

Chart 8.—The animals whose records are shown in Chart 8 derived their protein and water-soluble B from 50 per cent of cooked, dried, muscle tissue (beefsteak). The protein content of the diet of Lot 1236 was about 35 per cent. The growth curves approximated the average and the animals appeared to be in a satisfactory state of nutrition.

There were two females in Lot 1236. Together they had thirty-four young (five litters) and weaned twenty-four of them. One

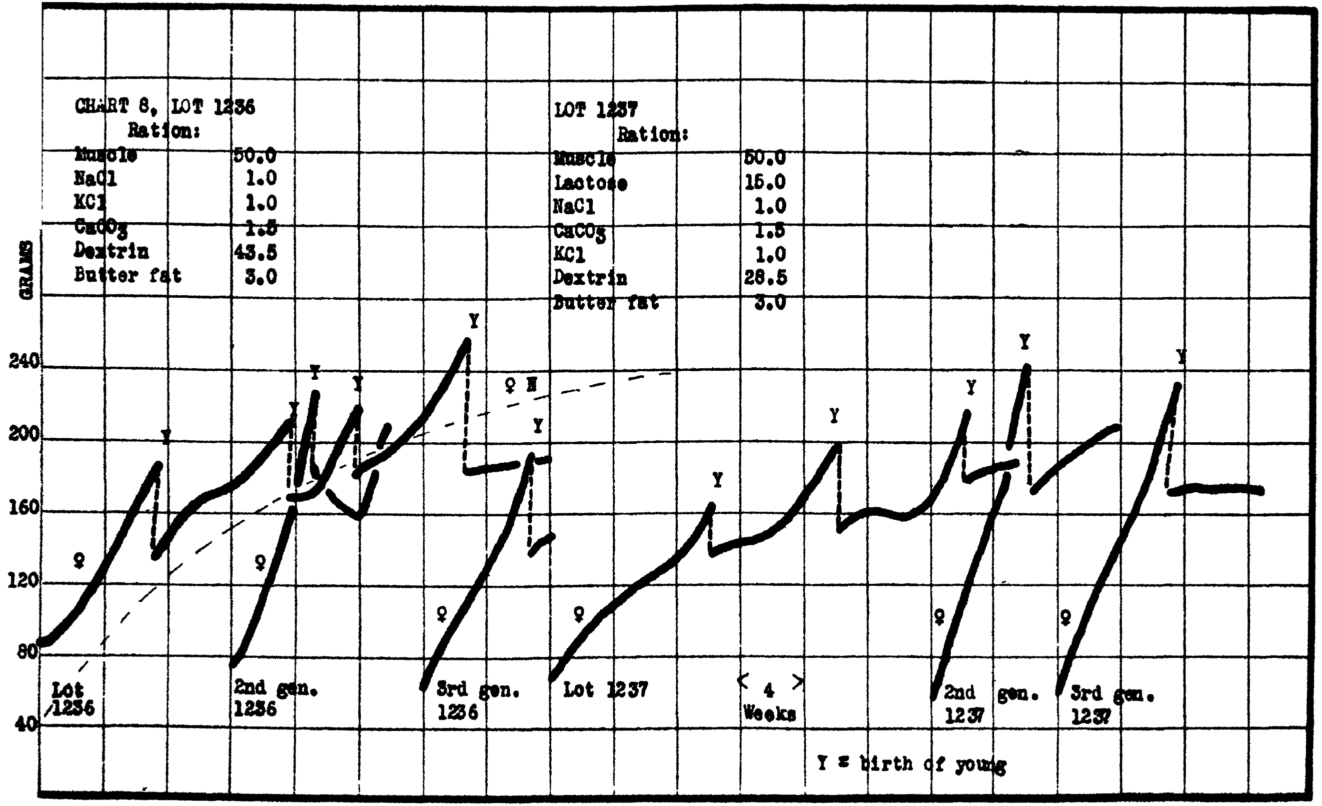
CHART 8, LOT 1236
Ration:
Muscle 50.0
NaCl 1.0
KCl 1.0
CaCO3 1.5
Dextrin 43.5
Butter fat 3.0
LOT 1237
Ration:
Muscle 50.0
Lactose 15.0
NaCl 1.0
CaCO3 1.5
KCl 1.0
Dextrin 28.5
Butter fat 3.0
GRAMS
240
200
160
120
80
40
Y
♀
♀ N
Lot 1236
2nd gen. 1236
3rd gen. 1236
Lot 1237
< 4 > Weeks
2nd gen. 1237
3rd gen. 1237
Y = birth of young

daughter of one of these mothers was kept on the diet. She had one litter of eight young and weaned them all. Two of her daughters grew up on the diet and each had a litter, together thirteen young, and weaned but three of them. The young in most cases appeared normal. They were not urine-stained as many were on the liver diets.

Lot 1237 differed from Lot 1236 in that 15 per cent of lactose replaced an equivalent amount of dextrin. There was no evidence that this carbohydrate exerted any beneficial effect over dextrin.

Two females in this group had together thirty young (six litters) and successfully weaned them all. Two of the second generation females had each a litter (collectively fifteen young), of which eleven young were weaned. One third generation female had a litter of seven young and weaned them all.

Chart 9.—These records show the histories of three groups of young rats fed diets in which all protein and antineuritic factors were derived from raw muscle tissue (round steak). Lot 2056 was restricted in the first period to raw round steak as the sole source of nutriment. They grew no better on this than on cooked steak (Chart 7, Lot 1232). In a second period sodium chloride and calcium carbonate were added and these caused a slight response with growth for a time. The diet was too poor in fat-soluble A to admit of much growth. This is seen in the record of Lot 2058.

Lot 2058 was fed a diet containing 50 per cent of raw, dried muscle (beefsteak) supplemented with sodium, potassium, calcium, chlorine, and fat-soluble A in butter fat. The diet contained 43.5 per cent of dextrin. On this ration growth was normal and the animals were apparently in good nutritive condition. Three females each had a litter of young (collectively twenty-two) of which twenty were reared. Two second generation females each had a litter (fourteen young) and reared them all.

There was a period between the ages of 18 and 28 days when the young of the second generation appeared lethargic. They recovered later and appeared to be as alert as rats on the cooked steak. This was probably due to the effects of eating so much raw meat while still in a very immature condition.

Lot 2057 had a diet like that of Lot 2058 except that 15 per cent of dextrin was replaced by a like amount of lactose. There was

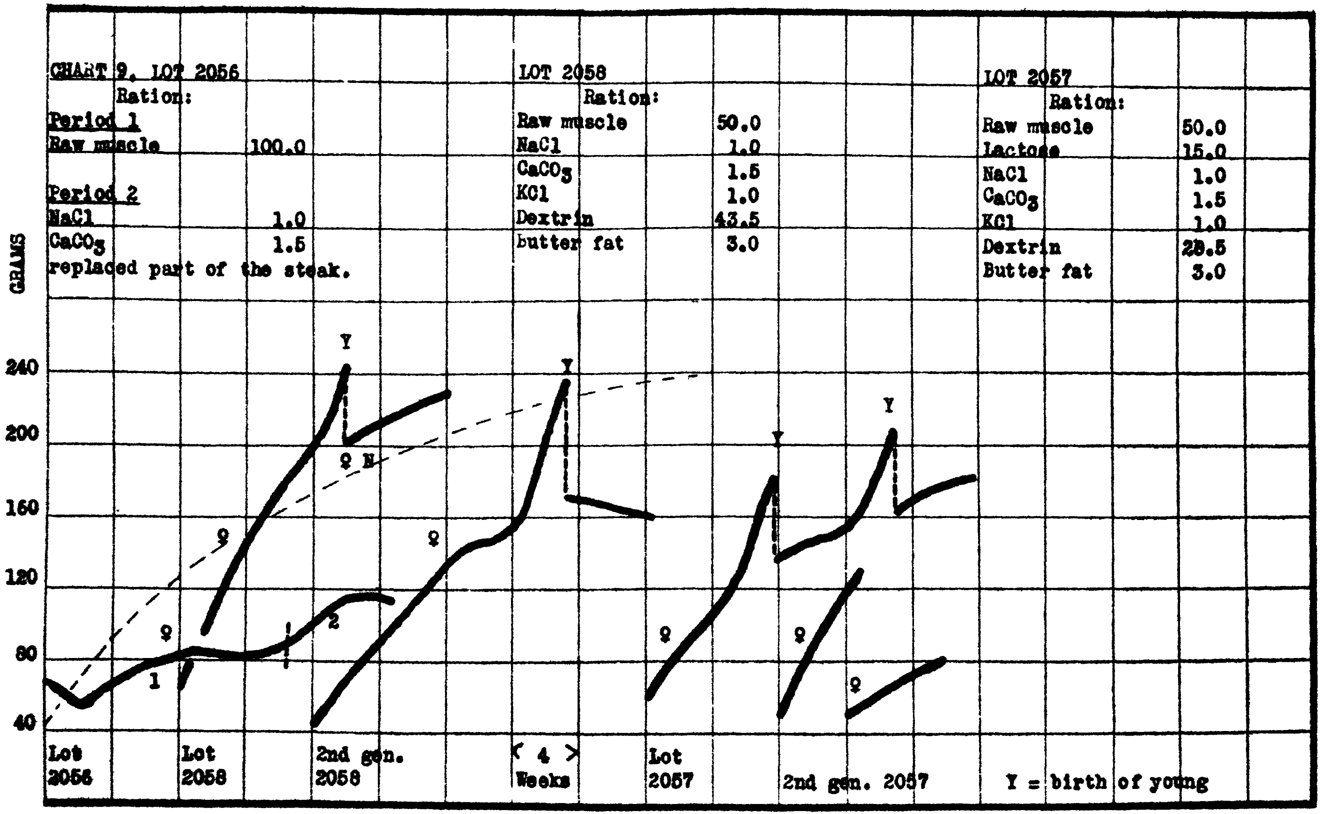

CHART 9. LOT 2056

Ration:	
Period 1	
Raw muscle	100.0
Period 2	
NaCl	1.0
$CaCO_3$	1.5

replaced part of the steak.

LOT 2058

Ration:	
Raw muscle	50.0
NaCl	1.0
$CaCO_3$	1.5
KCl	1.0
Dextrin	43.5
butter fat	3.0

LOT 2057

Ration:	
Raw muscle	50.0
Lactose	15.0
NaCl	1.0
$CaCO_3$	1.5
KCl	1.0
Dextrin	28.5
Butter fat	3.0

Y = birth of young

no noticeable benefit from this modification of the diet. There were two females in this group. They had collectively twenty-three young (four litters) of which twenty-two were weaned. No young have as yet been secured from any of the daughters. These records when compared with those of Chart 8 indicate that no noticeable difference exists in the nutritive value of raw and cooked steak. Although steak is very poor in water-soluble B, there was a sufficient amount of it in 50 per cent of cooked steak to permit young rats to grow up and rear young.

SUPPLEMENTARY PROTEIN VALUES IN FOODS.

II. SUPPLEMENTARY DIETARY RELATIONS BETWEEN ANIMAL TISSUES AND CEREAL AND LEGUME SEEDS.

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In a former publication[1] we have shown that the proteins of the navy bean do not markedly supplement those of the maize kernel so as to improve their quality for the support of growth. This means that the proteins of this cereal and legume seed are both deficient in some of the same indispensable amino-acids. Our earlier studies also indicated that the cereal proteins do not have much of a supplementary value among themselves.[2] In a later paper of this series we shall present more records bringing out the extent to which the proteins of the cereal grains and the legume seeds enhance each other's values for the nutrition of the rat. The data presented in the charts show the nutritive values of the protein mixtures resulting from combinations of the cereal grains and legume seeds with several representative animal tissues.

We recently pointed out the surprising fact that when muscle tissue, liver, or kidney, serves as the sole source of protein in the diet, all other factors in which have been made satisfactory by suitable additions, the quality of the amino-acid mixture for transformation into body proteins during growth is no better than that of proteins derived from grains.[3] It has always been accepted as a fact, hardly in need of experimental demonstration, that these animal proteins have exceptionally high biological values. Thomas came to this conclusion as the result of experimental data obtained on a full grown man as a subject. The periods of observation were very short, and a milk diet was used in the foreperiods which vitiated the results because of the supplementary effect on the foods being studied. Thomas' experimental data represent digestibility and absorbability of proteins rather than biological value for transformation into body tissue.[4]

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[1] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

[2] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

[3] McCollum, E. V., Simmonds, N. and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 111.

TABLE I.

Chart.	Lot.	Source of protein.	Growth.	Fertility.	Mortality.
2	2193	⅔ wheat, ⅓ kidney.	Excellent.	High.	Medium.
	2192	⅔ soy beans, ⅓ kidney.	Fair.	Very low	High.
3	2479	⅔ barley, ⅓ kidney.	Good.	High.	Medium.
4	2195	⅔ rolled oats, ⅓ kidney.	Fair.	Medium.	"
5	2194	⅔ maize, ⅓ kidney.	"	"	High.
	2191	⅔ navy beans, ⅓ kidney.	"	Low.	Low.
6	2190	⅔ peas, ⅓ kidney.	"	"	High.
	2196	⅔ rye, ⅓ "	Good.	Medium.	"

Of all the combinations of proteins from the two sources which we have studied, wheat and kidney proved to have the highest biological value. We have tested only one mixture of proteins from these sources; *viz.*, that in which a seed furnished two-thirds and kidney one-third of the total. Since in many cases growth was essentially normal, the basis of judgment in the comparison of the values of protein mixtures was the variation in fertility and infant mortality.

The combination of navy bean and kidney proteins forms a mixture having a higher biological value than any other animal tissue and legume seed which we have studied. Soy bean and kidney form a better protein mixture than soy bean with liver or soy bean and muscle.

The results of our studies show that the proteins of kidney, liver, and muscle have remarkable values for the enhancement of the proteins of some of the cereal grains, and improve the legume seed proteins to an appreciable degree. Kidney, liver, and muscle proteins have without exception supplemented those of the cereal grains more satisfactorily than they do those of the legume seeds. There are marked differences in the efficiency of the supplementary relations between kidney proteins and those of several cereal grains which make it possible for us to differentiate between their values in the case of the different combinations. The relative values of the several combinations of vegetable proteins with those of kidney with which we have experimented in this series may be illustrated by tabulating the seeds in the order of their efficiency for growth, when two-thirds of the protein is derived from the seed and one-third from kidney.

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[4] Thomas, K., *Arch. Physiol.*, 1909, 219.

Wheat—barley—rye—oat—maize—soy bean—pea—navy bean.

There is no marked differentiation between the kidney and oat as contrasted with the kidney and maize combinations. These are distinctly inferior to the combinations of kidney with barley, wheat, or rye. There is little difference in the biological values of the mixtures of proteins derived from peas or the two kinds of beans studied. These legume seeds combined with animal tissues are inferior to similar combinations of animal tissues with cereals. Table I shows in condensed form the effects of feeding kidney proteins as supplements to certain vegetable proteins on growth, fertility, and infant mortality.

Among the several combinations of cereal and legume seed proteins with muscle tissue which we have studied, wheat is decidedly the best. Growth and fertility were remarkable, but the infant mortality was high. Muscle tissue is distinctly less effective than kidney as a supplement to the proteins of the seeds used, except in the case of wheat. The following order represents the biological values of combinations of seed with muscle in which the seed furnished two-thirds of the total protein and the muscle one-third. The values are arranged in a descending scale.

Wheat—oat—barley—maize—pea—navy bean—soy bean.

It is not possible to differentiate from our data between the values of navy bean and soy bean proteins combined with muscle. Table II shows in condensed form the effects on growth, fertility, and infant mortality of feeding muscle proteins as supplements to the proteins of several seeds.

Liver proteins supplement those of the cereal grains and legume seeds in degrees which differ sufficiently to make possible rather decided contrasts in several cases. In general, liver proteins enhance those of the seeds somewhat better than do those of muscle, with the single exception of the combination, wheat and muscle. The seeds which we have studied may be arranged in the following order to illustrate the supplementary efficiency of liver proteins for each, the best combination being on the left and decreasing from left to right.

Barley—rye—wheat—oat—maize.

TABLE II.

Chart	Lot	Source of protein.	Growth	Fertility	Mortality
7	2186	⅔ wheat, ⅓ muscle.	Excellent.	High.	High.
8	2189	⅔ rye, ⅓ "	Very good.	"	"
	2184	⅔ navy beans, ⅓ muscle.	Poor	Low.	"
9	2188	⅔ rolled oats, ⅓ "	Good.	High.	"
	2477	⅔ barley, ⅓ muscle.	"	Low.	"
10	2187	⅔ maize, ⅓ "	Fair.	"	"
	2183	⅔ peas, ⅓ "	"	"	"
	2185	⅔ soy beans, ⅓ muscle.	"	"	"

Wheat and muscle gave an excellent growth curve and high fertility but apparently because of a slight inferiority of this protein mixture as compared with certain others, the infant mortality was high. Wheat and muscle appear to form a protein mixture superior to rye and muscle. The growth curves of rats confined to a protein supply derived from rolled oats and muscle were better than the curves of those fed rolled oats and kidney. The latter, however, were more successful in fertility and in rearing young than the former.

It is not possible from our data to decide between the values of the combinations of liver proteins with those of the legume seeds, peas, navy beans, and soy beans. A study of the records presented here, showing the effects of seed and meat diets, supplemented with respect to all factors other than protein, on the life histories of individual animals and on the family histories of successive generations confined to monotonous food mixtures, cannot fail to impress one with the fact that observations on the growth curves of a group of animals to maturity do not afford sufficient evidence to enable one to arrive at safe deductions concerning the quality of an experimental diet. Table III shows the effect on growth, fertility, and infant mortality, of feeding liver proteins as supplements to the proteins of several seeds.

There are many who believe that if the diet affords sufficient calories and considerable variety, satisfactory nutrition will be assured. This may be far from true. So long as the selection does not include a variety of types of foods of unlike dietary values variety does not insure safety in any great degree. No matter how many cereals, tubers, and muscle meats such as steak, ham, chops, roasts, etc., may be taken, the diet will prove to be inadequate. The inclusion of milk or leafy vegetables goes far toward making good deficiencies of such a diet in inorganic constituents and fat-soluble A. Fresh fruits and uncooked salad vegetables have a unique place in the diet as sources of the antiscorbutic factor water-soluble C.

TABLE III.

Chart.	Lot.	Source of protein.	Growth.	Fertility.	Mortality.
11	2478	⅔ barley, ⅓ liver.	Good.	High.	Medium.
	2178	⅔ soy beans, ⅓ liver.	Poor.	Low.	High.
12	2180	⅔ maize, ⅓ liver.	Fair.	Medium.	"
13	2181	⅔ rolled oats, ⅓ liver.	Good.	High.	"
14	2182	⅔ rye, ⅓ liver.	Very good.	"	"
	2176	⅔ peas, ⅓ "	Fair.	Low.	"
15	2179	⅔ wheat, ⅓ "	Good.	High.	"
	2177	⅔ navy beans, ⅓ liver.	Fair.	Low.	"

Rolled oats and liver formed a better protein mixture than rolled oats with kidney or muscle. This could not be discerned from an inspection of the growth curves or the records of fertility, but came to light through the comparison of the infant mortality in the three groups.

Barley and liver made a better protein mixture than barley and muscle, but not quite so good as barley and kidney. Rye and liver also proved superior as a source of protein to mixtures of this cereal with either muscle or kidney.

Navy beans with liver form a better protein mixture than combinations of this seed with muscle, but not quite so good as with kidney.

The substitution at frequent intervals of ham for chops will not tend to insure safety in nutrition. So large a part of the food supply of man in the temperate and warmer regions of the world is derived from cereals and other seed grains, tubers, and edible roots, that in seeking variety in food attention should be fixed upon the importance of milk and the leafy vegetables suitable for greens, salads, etc., since these are so constituted as to correct the deficiencies of cereals, tubers, and roots.[5]

In examining the records of the experimental animals described in this paper it should be kept in mind that in studying the combinations of proteins from animal and vegetable sources, we have in all cases added butter fat to furnish fat-soluble A and the inorganic salts necessary to correct the mineral deficiencies. The results do not, therefore, represent the supplementary effect of one of these foodstuffs for another except in respect to the protein factor.

The results of these studies form a demonstration of the value of animal tissues as constituents of the diet, when the latter contains as much cereal products as is usually the case in America and Europe at the present time. At the same time it must be remembered that the animal tissues are by no means complete supplements for a cereal and legume seed diet.

SUMMARY.

1. The proteins of kidney, liver, and muscle are remarkably effective as supplements for the proteins of cereals. There are demonstrable differences in the extent to which these animal tissues enhance the values of certain of the cereals. Thus, kidney, liver, and muscle are about equally effective as supplements to wheat. Maize proteins are less effectively supplemented by kidney, liver, or muscle than wheat, barley, or rye.

2. The three animal tissue proteins studied were found to have a supplementary relation to pea, navy bean, or soy bean proteins, but these combinations are very inferior to similar combinations of animal tissues with cereal grains as a source of protein.

3. Our results demonstrate that the proteins of kidney, liver, or muscle are more valuable for transformation into body tissues when combined with cereal proteins, than when each is fed as the sole source of amino-acids in the diet.[3]

4. Either muscle or glandular substance fails to supplement effectively the mineral deficiencies of cereals or legume seeds. The glandular organs are good sources of fat-soluble A.

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[5] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113.

Chart 1.—These curves show typical records of growth and reproduction of rats fed from an early age on diets in which the sole source of protein was a cereal or legume seed. The amount of seeds was adjusted in each case so as to give the diets a protein content of 9 per cent. Such additions of butter fat and inorganic salts were made in each case as to correct dietary deficiencies other than protein of these seeds.

The list of cereals here discussed includes wheat, maize, rolled oats, barley, and the legumes, peas, navy beans, and soy beans.

Among these seeds wheat stands first in quality of protein when fed as the sole source of this factor. Oat and maize proteins have approximately the same value, but we have gained the impression that oat proteins are slightly superior. None of the legume seeds is at all satisfactory as the sole source of protein in the diet at the plane of intake of 9 per cent of the food mixture. This fact is brought out by the curves of Lots 2376, 2510, and 2368. On adding casein in a second period growth was accelerated in each case.

No young have been secured on diets of this type in which proteins were derived entirely from a legume seed or from maize. The fertility of rats fed oats to furnish 9 per cent of protein was so far below that of animals fed wheat protein in similar amounts as to indicate that the latter were in a distinctly better state of well being. Even in the case of the wheat diet the protein was not sufficiently good to permit of much success in rearing young. A few young could be reared by mothers which had grown up and were maintained on the oat diet containing 9 per cent of protein.

These curves are presented as a basis of comparison for the records in the remaining charts described in this paper. These derive their content of protein (9 per cent) in each case from two sources. The object in each case is to compare the biological value of mixtures of proteins in stated proportions as compared with the values of each source when fed alone.

Chart 2.—Lot 2193 was fed a diet from soon after weaning, which derived its protein content of 9 per cent from wheat and cooked dry kidney. All other factors in the diet were adequately supplemented. The wheat furnished 6 per cent and the kidney 3 per cent of the protein of the diet. Growth was normal in this group. The state of nutrition of these animals was much superior

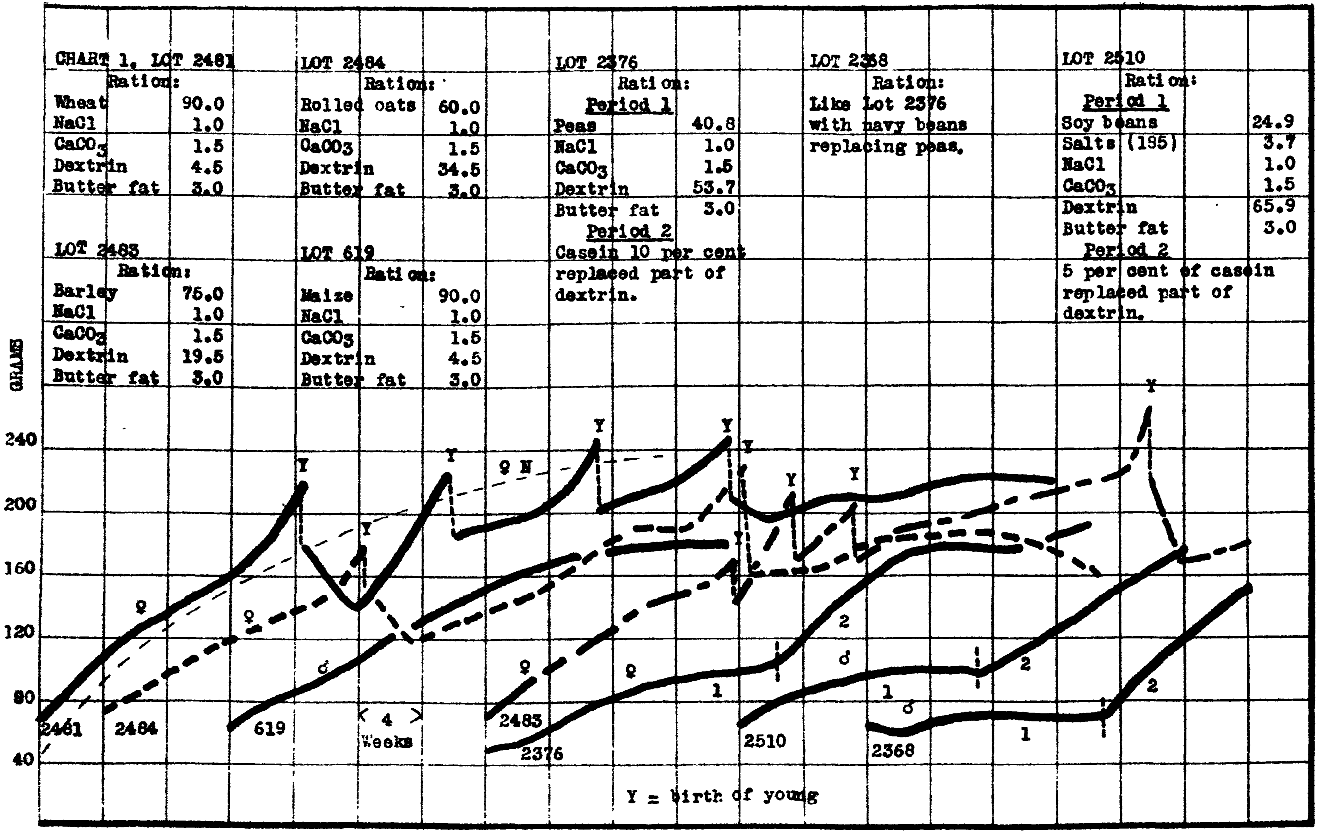
CHART 1. LOT 2481
Ration:
Wheat 90.0
NaCl 1.0
CaCO3 1.5
Dextrin 4.5
Butter fat 3.0
LOT 2483
Ration:
Barley 75.0
NaCl 1.0
CaCO3 1.5
Dextrin 19.5
Butter fat 3.0
LOT 2484
Ration:
Rolled oats 60.0
NaCl 1.0
CaCO3 1.5
Dextrin 34.5
Butter fat 3.0
LOT 619
Ration:
Maize 90.0
NaCl 1.0
CaCO3 1.5
Dextrin 4.5
Butter fat 3.0
LOT 2376
Ration:
Period 1
Peas 40.8
NaCl 1.0
CaCO3 1.5
Dextrin 53.7
Butter fat 3.0
Period 2
Casein 10 per cent replaced part of dextrin.
LOT 2368
Ration:
Like Lot 2376 with navy beans replacing peas.
LOT 2510
Ration:
Period 1
Soy beans 24.9
Salts (185) 3.7
NaCl 1.0
CaCO3 1.5
Dextrin 65.9
Butter fat 3.0
Period 2
5 per cent of casein replaced part of dextrin.
GRAMS
240
200
160
120
80
40
Y
♀
♂
♀ N
1
2
2481
2484
619
2483
2376
2510
2368
< 4 >
Weeks
Y = birth of young

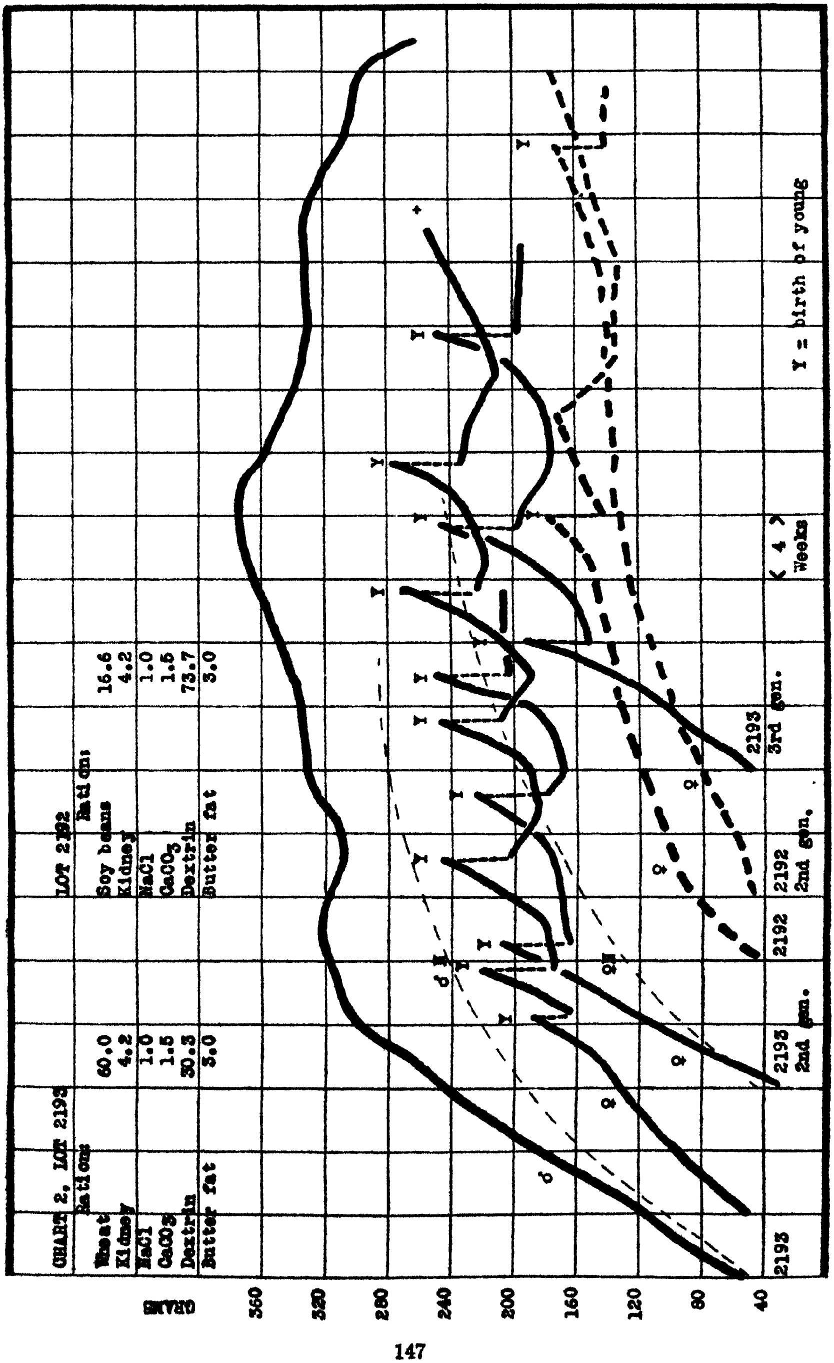
CHART 2. LOT 2193
Rations:
Wheat 60.0
Kidney 4.2
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 30.3
Butter fat 3.0
LOT 2192
Rations:
Soy beans 16.6
Kidney 4.2
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 73.7
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
2193
2193 2nd gen.
2192
2192 2nd gen.
2193 3rd gen.
< 4 > Weeks
Y = birth of young
♂
♀
Y

to those fed wheat as the sole source of protein when the content of the latter was limited to 9 per cent. Those rats which grew up on 9 per cent of wheat protein (Lot 2481, Chart 1) were as old looking at 14 months as were those in Lot 2193 at 18 to 20 months. Indeed, the animals of Lot 2193 were in such good condition that they compared favorably in appearance and in fertility with other experimental groups fed much higher protein intakes.

There were two females in this group. They had collectively seventy-nine young (eleven litters) and weaned seventy of them. On a diet comparable in all respects but with all its 9 per cent of protein derived from wheat we observed 50 per cent mortality of the young. A somewhat higher protein content in the diet of Lot 2193 would apparently have shortened the nursing periods, which were longer than normal, and would have lowered the infant mortality. Four females in the second generation had seventy-five young (twelve litters) and weaned forty-nine of them. Two third generation females had forty-nine young (eight litters) and weaned thirty-six. The mortality of the young in this group was due entirely to destruction by the mothers. Frequently the young were bitten and injured or killed when approaching the end of the nursing period.

One cannot say that animals, which had a diet containing enough protein to enable them to grow at the normal rate to maturity and to be as fertile as were these, were suffering from protein starvation. Yet it was due to the low protein intake (of the quality here used) which exerted a peculiar influence on the psychological reactions of the mothers and made them attack their young even after they were 2 or 3 weeks old. In rats in our colony which have highly satisfactory diets it is extremely rare that a mother attacks her offspring or indeed those of another. We not infrequently isolate among our stock animals two or three pregnant females in the same cage. They then care for their young in a common nest and nurse their own young and those of the other mothers promiscuously and without perversion of the maternal instinct.

Lot 2192 was fed a diet in which the protein was furnished by soy beans and beef kidney. The diet contained 9 per cent of total protein, two-thirds of which came from soy beans and one-third from kidney. All the other factors were satisfactorily supplemented. Growth was slow and the animals never reached full adult size. Their growth was much better, however, than that of rats restricted to the same amount of protein entirely from soy beans (Chart 1, Lot 2510).

Two females grew up on this diet. One remained sterile. The other had nine young (two litters) and weaned three. Two second generation females remained sterile. The animals in this group were very old looking at 15 months of age. It is evident that 9 per cent of protein from these sources is not satisfactory for normal milk production.

Chart 3.—Lot 2479 was fed a diet in which the protein was derived from barley and kidney. The barley furnished two-thirds and the kidney one-third of the total protein. The growth was more satisfactory than any we have observed in rats fed 9 per cent of barley protein only (see Chart 1, Lot 2483).

Two females which grew up on this diet had collectively forty-five young (seven litters) and weaned thirty-one. One female in the second generation had nineteen young (three litters) and weaned eighteen. Two third generation females had forty-three young (seven litters) and weaned nineteen. One fourth generation female was restricted to the diet but she failed early and died at the age of 110 days. These animals were in good condition at the age of 15 months, when they were discarded.

Chart 4.—Lot 2195 was fed a diet in which the protein was furnished entirely by rolled oats and kidney The oats furnished two-thirds and the kidney one-third of the total protein. Their records were very much better than those of Lot 2484 (Chart 1). This is shown especially in the fertility and infant mortality of the two groups. Lot 2195 was somewhat undersized but of good appearance. They were not in such good condition as those which grew up on the wheat and kidney diet (Lot 2193, Chart 2).

Two females grew up on this diet. One of these had twenty young (four litters) and weaned ten. The other died soon after giving birth to her first litter of six young. Two second generation females had thirty-seven young (six litters) and weaned twenty-three. One third generation female had one litter of five and weaned them all. These young were in good condition, but the nursing periods were long. The animals in this group were old looking at about 16 months.

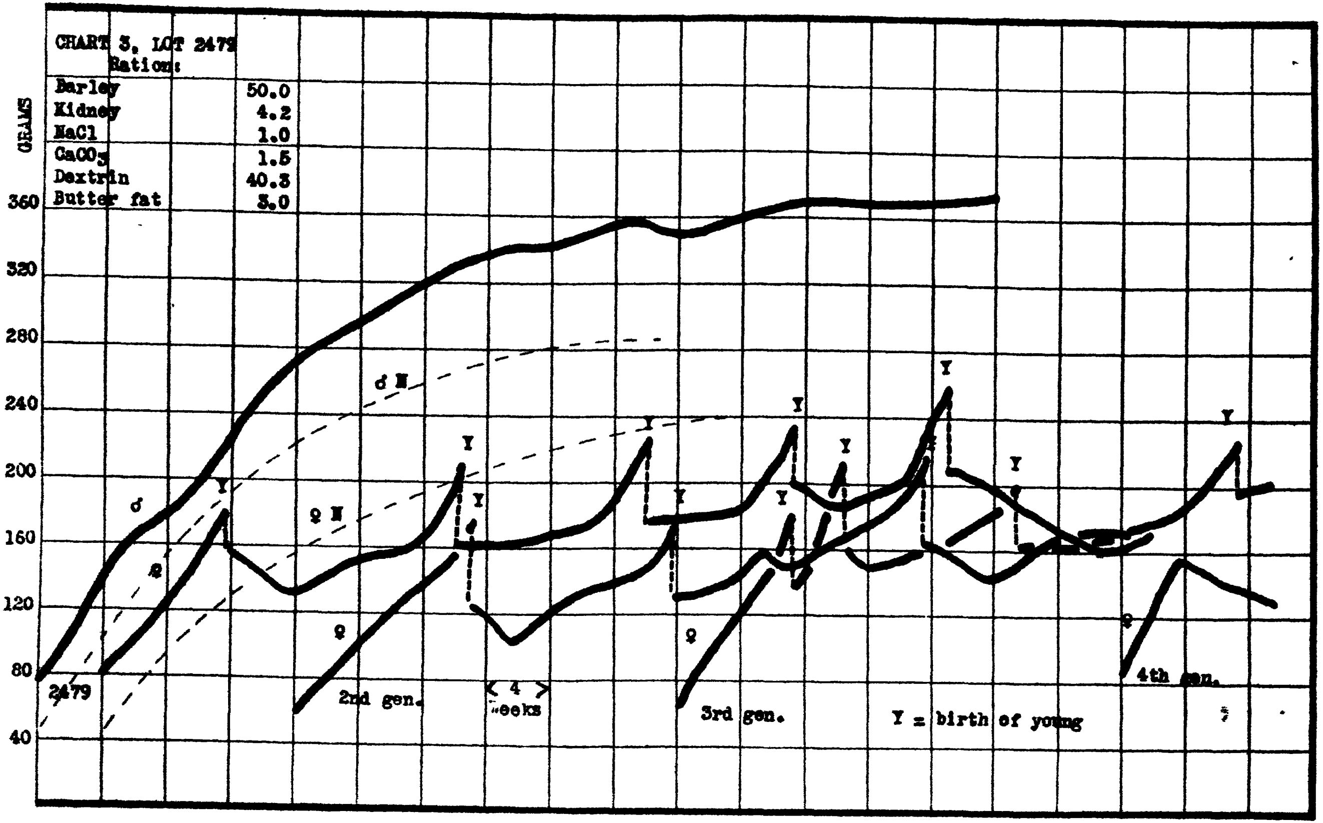
CHART 3, LOT 2479
Ration:
Barley 50.0
Kidney 4.2
NaCl 1.0
CaCO3 1.5
Dextrin 40.3
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
♂
♀
2479
♂ N
♀ N
Y
2nd gen.
< 4 >
weeks
3rd gen.
Y = birth of young
4th gen.

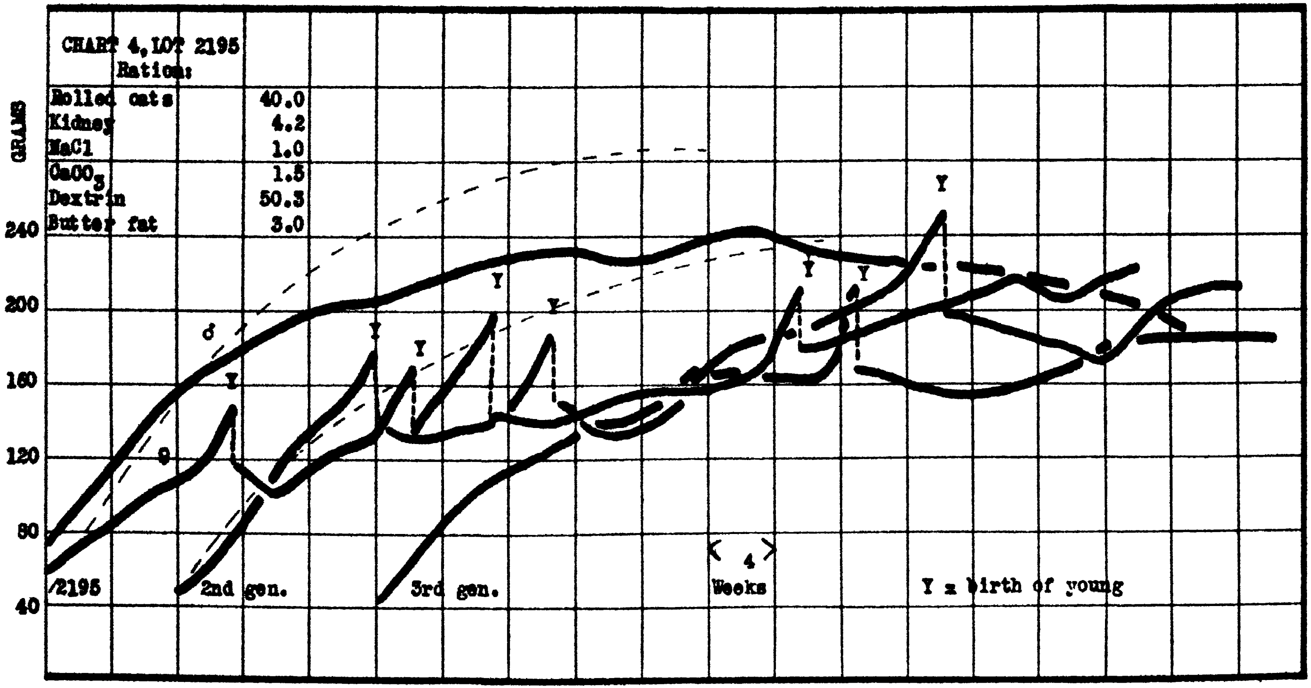
CHART 4, LOT 2195
Ration:
Rolled oats 40.0
Kidney 4.2
NaCl 1.0
CaCO3 1.5
Dextrin 50.3
Butter fat 3.0
GRAMS
240
200
160
120
80
40
♂
♀
Y
/2195
2nd gen.
3rd gen.
< 4 >
Weeks
Y = birth of young

Chart 5.—Lot 2194 was fed a diet, the protein of which was derived entirely from maize and kidney. The maize furnished two-thirds of the total protein and the kidney one-third. All other factors were satisfactorily supplemented. The growth curves were much better than were those of Lot 619 (Chart 1), which had a diet containing 9 per cent of maize protein alone. The animals of Lot 2194 on the maize and kidney proteins were never in good condition, but did not look very aged until about 18 months. Three females which grew up on this diet had thirty-one young (six litters) and weaned fifteen. They were only in fair condition and were small for their ages. One second generation female grew up on the diet and had fifteen young (three litters). She weaned four of her third litter. The other young she destroyed soon after they were born.

Lot 2191 was fed a diet containing 9 per cent of protein, two-thirds of which was furnished by navy beans and one-third by kidney. All factors other than protein were satisfactorily supplemented. A diet exactly similar but with 9 per cent of protein derived solely from navy beans supports almost no growth (Lot 2368, Chart 1). The combination of navy beans and kidney is therefore vastly superior to bean protein alone. These animals looked very old at about 12 months.

One female in this group had one litter of five young and weaned four, but they were very small. At 42 days of age the four weighed but 147 gm. A litter of four nursing a normally fed mother should reach a weight of about 350 gm. at this age. One other female in this group remained sterile. Two second generation females were restricted to the diet but neither one had any young although their growth curves were much better than those of rats confined to 9 per cent of navy bean protein alone (Lot 2368, Chart 1).

Chart 6.—Lot 2190 was fed a diet containing 9 per cent of protein, two-thirds of which was furnished by peas and one-third by kidney. All other factors were satisfactorily supplemented. The animals grew much better than a group confined to a similar diet with 9 per cent of pea protein as its sole source of nitrogen (Lot 2376, Chart 1). This shows that there is a greatly heightened value in the combination of proteins used in this diet as compared with pea protein alone. The rats fed this ration were somewhat

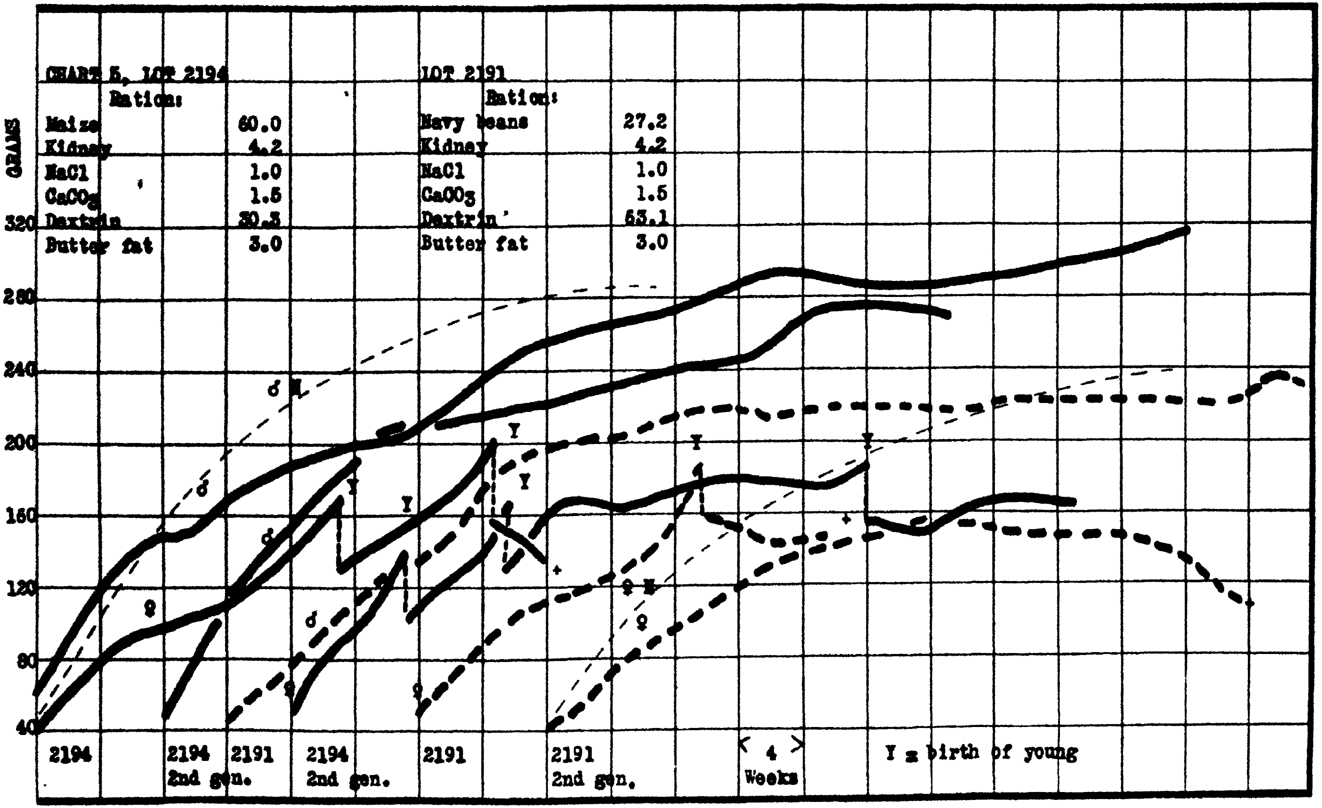
CHART 5, LOT 2194
Ration:
Maize 60.0
Kidney 4.2
NaCl 1.0
CaCO3 1.5
Dextrin 30.3
Butter fat 3.0
LOT 2191
Ration:
Navy beans 27.2
Kidney 4.2
NaCl 1.0
CaCO3 1.5
Dextrin 63.1
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
♂
♀
Y
2194
2194 2nd gen.
2191
2194 2nd gen.
2191
2191 2nd gen.
< 4 > Weeks
Y = birth of young

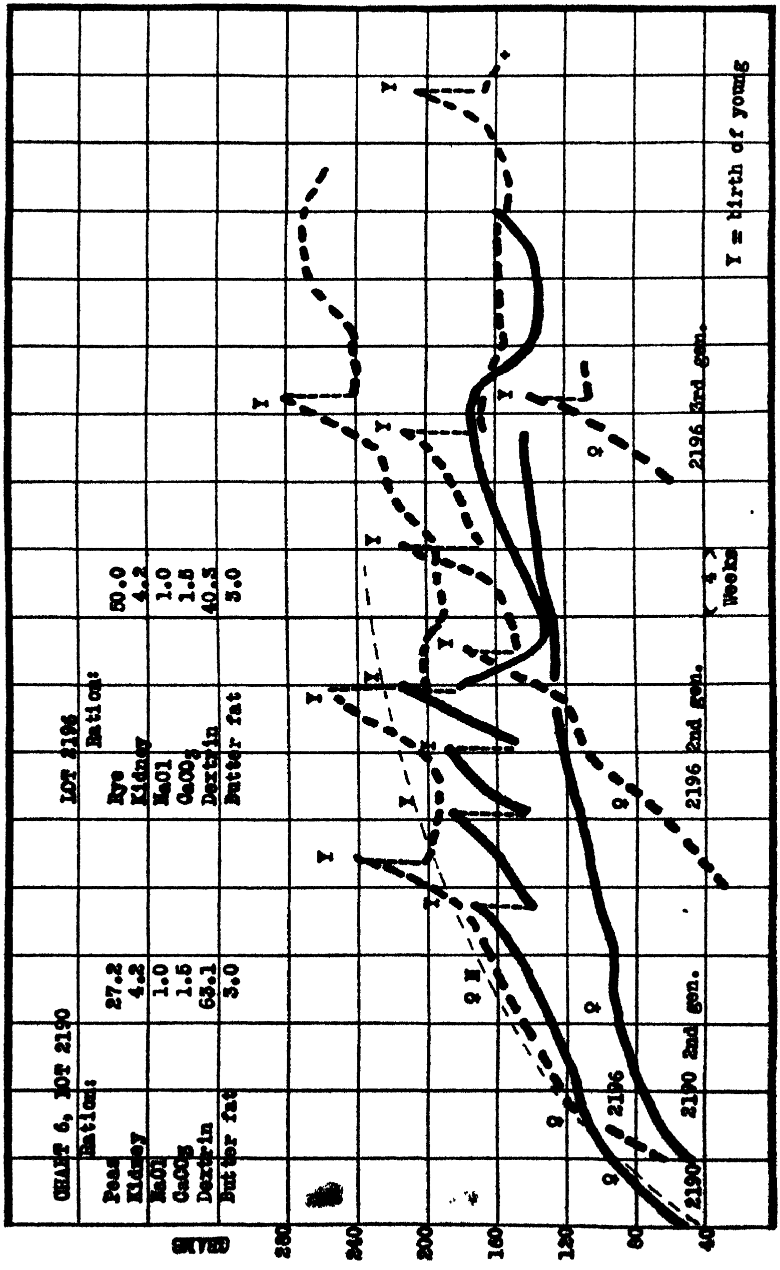
CHART 6, LOT 2190
Ration:
Peas 27.2
Kidney 4.2
NaCl 1.0
CaCO3 1.5
Dextrin 63.1
Butter fat 3.0
LOT 2196
Ration:
Rye 50.0
Kidney 4.2
NaCl 1.0
CaCO3 1.5
Dextrin 40.3
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
Y = birth of young
4 Weeks
Y
♀
♀ H
2190
2196
2190 2nd gen.
2196 2nd gen.
2196 2nd gen.

undersized, but appeared fairly well nourished in early life. They appeared senile when about 12 months of age.

One female in this group had twenty-four young (four litters) and weaned four of her fourth litter. The other young were destroyed by the mother while very young. Another female remained sterile. Three second generation females were confined to the diet until they were 12 months old, but none of them had any young.

Lot 2196 derived its sole protein from rye and kidney. The rye furnished two-thirds and the kidney one-third of the total. Except for the amount and quality of the protein the diet was well constituted. Although the growth of these animals was excellent and the fertility fairly high, the mortality of the young was very high. These rats were in good condition in early life but looked very old at about 16 to 18 months.

Five females on this diet had collectively seventy-three young (fourteen litters) and weaned thirty-one. Many of these were destroyed by the mothers soon after birth. One female died after the first litter, and a second died after her second litter from undetermined causes. Two second generation females had thirty-one young (seven litters) and weaned nine. One third generation female had a litter of six.

Our investigations show that cereal grain proteins are distinctly better supplemented by kidney proteins than are those of the legume seeds. The order of effectiveness in this supplementary relation is as follows:

Wheat—barley—rye—oat—maize.

There is no marked difference in the value of peas, soy beans, and navy beans in their supplementary relation to kidney proteins.

Chart 7.—Lot 2186 was fed a diet comparable to those discussed in Charts 2 to 6 inclusive, except that muscle tissue (beefsteak) replaced kidney as a supplemental source of protein for a seed. This diet contained 9 per cent of protein, two-thirds of which was furnished by wheat and one-third by beef muscle. Deficiencies in inorganic salts and fat-soluble A were made good by suitable additions.

The growth curves of this group, considering that the food mixture contained but 9 per cent of protein, are remarkable. All the

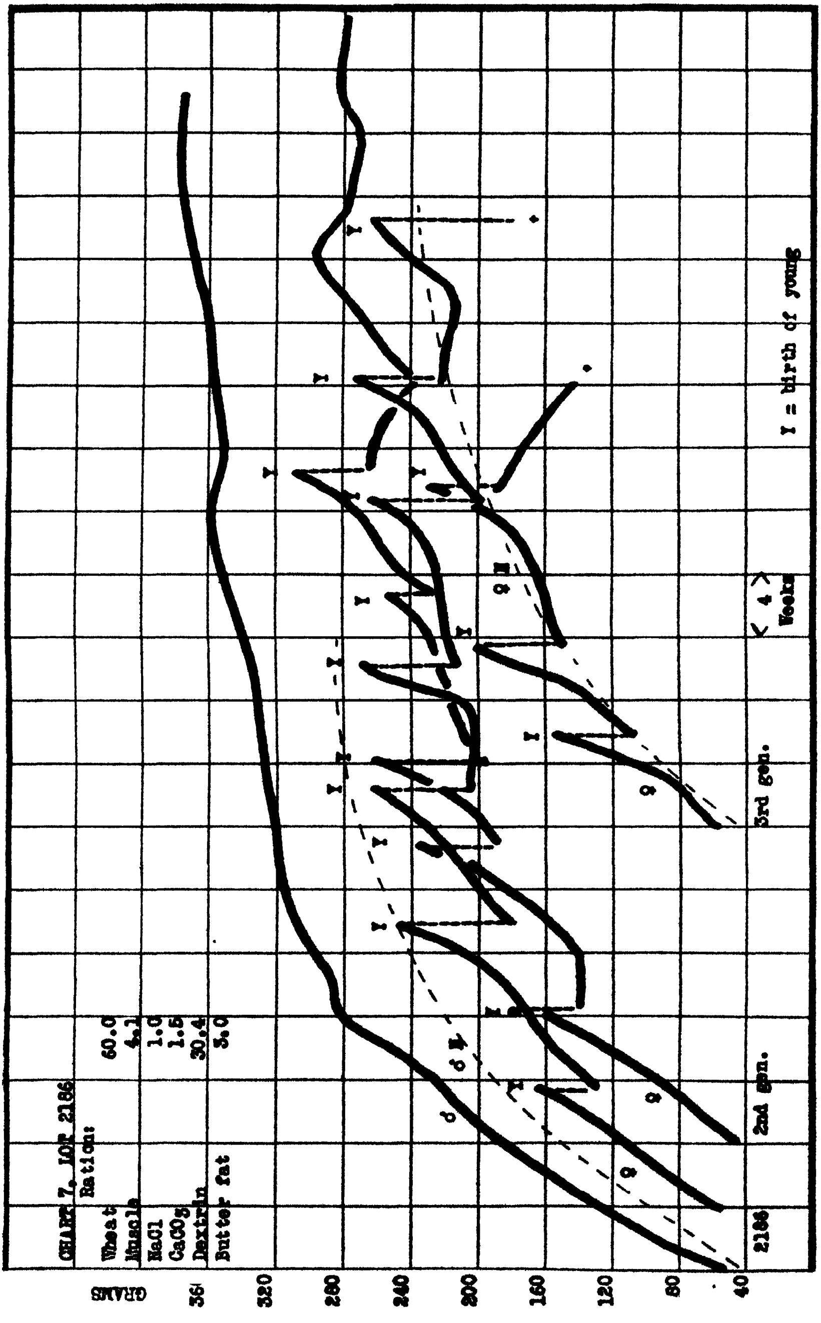
CHART 7. LOT 2166
Ration:
Wheat 60.0
Muscle 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 30.4
Butter fat 3.0
GRAMS
36
320
280
240
200
160
120
80
40
2166
2nd gen.
3rd gen.
< 4 >
Weeks
Y = Birth of young
Y
♂
♀
♂ M
♀ M

animals grew at a rapid rate to a large size. This can be interpreted only to mean that there is an excellent supplementary relation between the proteins of the wheat kernel and those of muscle tissue. This was likewise true of wheat supplemented with kidney (Lot 2193, Chart 2). The span of life was, however, somewhat short. The animals looked old at 16 to 17 months, whereas they remain vigorous up to 2 years at least when the nutrition is highly satisfactory. That they were not in this optimal condition, notwithstanding their large size and splendid fertility, is shown by the high mortality among their young. Although the biological value of the protein was high and the plane of intake sufficient to support growth and reproduction, the amount of protein of this quality was not good enough to maintain physiological well being to an advanced age or to suffice for the nutrition of the nursing young.

Two females grew up on this diet. They had collectively sixty young (eight litters) of which only twenty-nine were weaned. Two females of the second generation had sixty-five young (eleven litters) and weaned twenty-three. One third generation female had nineteen young (three litters) and weaned only three. Many of the young were destroyed shortly after birth, but those which were weaned were in good condition. In several instances the mothers reared large litters (nine young; seven young). This shows that even 9 per cent of protein of the excellent quality formed by wheat and muscle in the proportions here used sufficed for milk production even when the litters were large. We differentiate between high infant mortality due to infanticide and that due to undernutrition of the nursing young. It is most remarkable that rats on certain types of faulty diets should fail to exhibit the normal maternal instinct, which is very strong in this species.

Chart 8.—Lot 2189 was fed 9 per cent of protein in the form of rye and muscle. The cereal furnished two-thirds and the muscle one-third. The condition of these animals was not so good as that of Lot 2186 (Chart 7) fed a similar diet with the protein derived from wheat and muscle. They grew senile at about the same age as did Lot 2186.

Two females had collectively thirty-nine young (seven litters) and weaned fifteen. Two females of the second generation had forty-four young (eight litters) and weaned fifteen. Two third

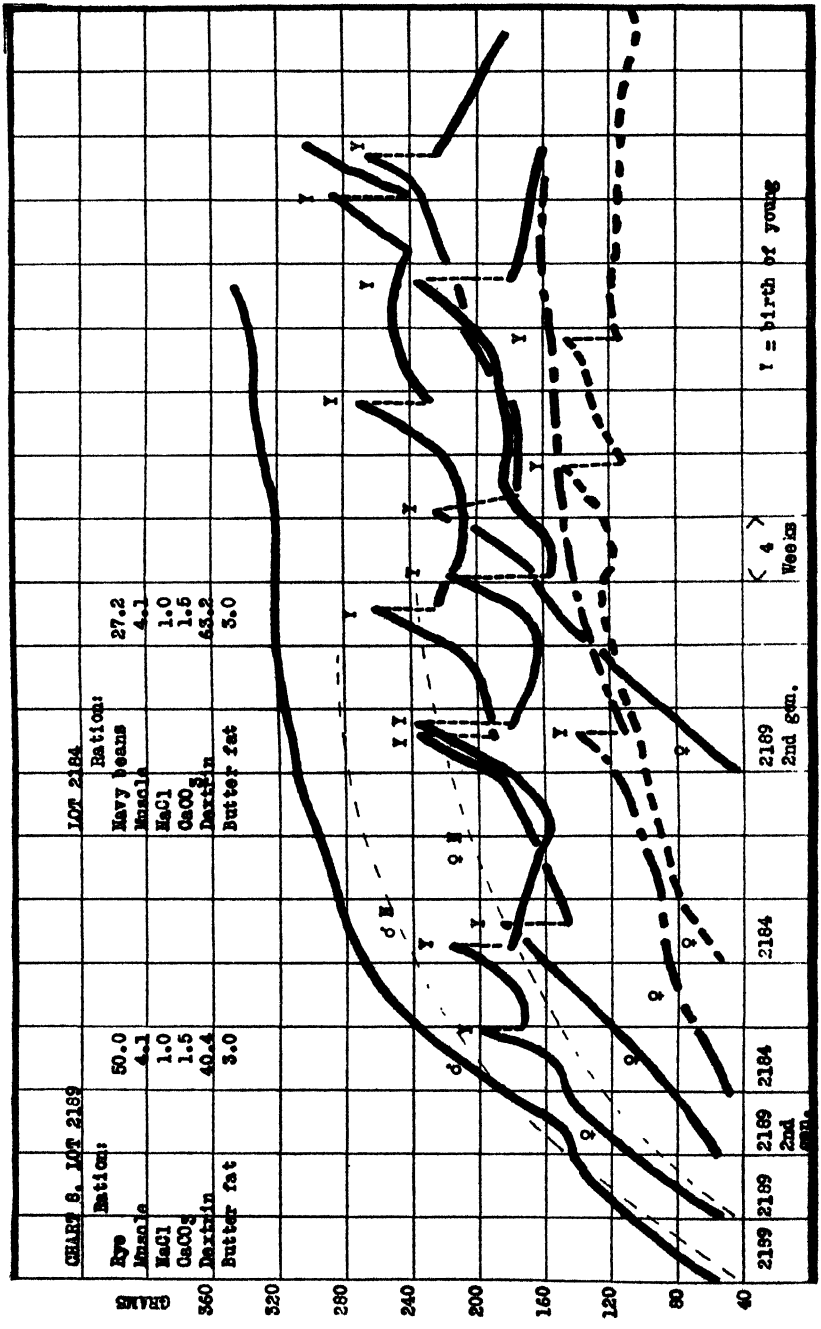
CHART 8. LOT 2189
Ration:
Rye 50.0
Muscle 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 40.4
Butter fat 3.0
LOT 2184
Ration:
Navy beans 27.2
Muscle 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 63.2
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
2189
2189
2189 2nd gen.
2184
2184
2189 2nd gen.
< 4 > Weeks
Y = birth of young
♂ M
♀ M
♂
♀
Y

generation females, had each a litter (eleven young) and weaned six. One third generation female remained sterile. The young of all generations always presented an inferior appearance. The nursing periods were very long and in some cases the young remained undersized. In one instance two young which survived to the 41st day were still nursing and weighed but 60 gm. together.

Lot 2184 was fed 9 per cent of protein in the form of navy beans (6 per cent) and muscle tissue (3 per cent). Other factors than protein were satisfactory. These rats grew poorly and their fertility was low. The growth on this diet was better than we have seen on 9 per cent of navy bean protein alone. They were very old looking at 15 months.

Two females had collectively ten young (three litters) and destroyed them all shortly after birth.

Chart 9.—Lot 2188 was fed a diet in which the total protein content was 9 per cent, two-thirds of which was furnished by rolled oats and one-third by muscle tissue. The growth curves were much better than on 9 per cent of oat protein alone (Chart 1, Lot 2484), and the animals remained longer in a good condition. They were old looking at 15 to 16 months of age. Oat and muscle proteins are not of so good quality as a similar mixture derived from wheat and muscle, but of about the same value as a mixture derived from maize and muscle. This is shown by a comparison of Lot 2188, Chart 9, with Lot 2186, Chart 7, and Lot 2187, Chart 10, both with respect to the growth curves and reproduction records.

There were two females in this group. They had seven litters (thirty-seven young) and killed them all. The young were destroyed by the mothers soon after birth. Another litter was born but destroyed before the number was ascertained.

Lot 2477 was restricted to a diet containing 9 per cent of protein. Barley furnished 6 per cent and muscle tissue 3 per cent. All other factors were made satisfactory by suitable additions. Their growth and ability to rear young were inferior to Lot 2186 (Chart 7) which was fed wheat and muscle tissue. They were very old looking at 14 months of age.

Three females in this group had collectively seventy-five young (seven litters) and weaned only two. After these two were weaned

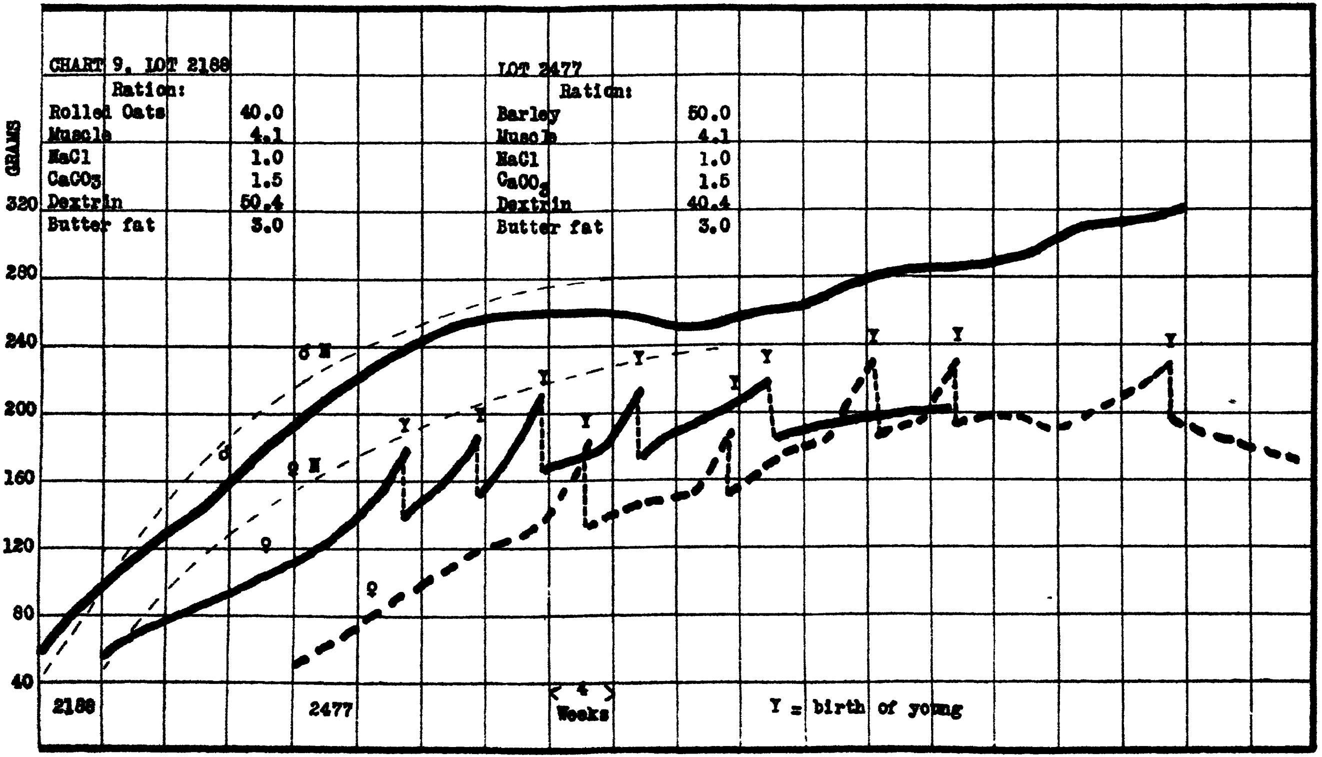
CHART 9. LOT 2188

Ration:	
Rolled Oats	40.0
Muscle	4.1
NaCl	1.0
$CaCO_3$	1.5
Dextrin	50.4
Butter fat	3.0

LOT 2477

Ration:	
Barley	50.0
Muscle	4.1
NaCl	1.0
$CaCO_3$	1.5
Dextrin	40.4
Butter fat	3.0

and returned to the family cage they were killed by the older animals.

Chart 10.—Lot 2187 was fed 6 per cent of maize protein and 3 per cent of muscle protein. Inorganic and fat-soluble A additions were made to correct the deficiencies in the maize and muscle mixture. The growth curves of this lot were distinctly below the optimum and inferior to the group fed wheat and muscle proteins. At 16 to 17 months they were very old looking and their offspring all presented an inferior appearance.

Two females had collectively thirty-three young (six litters) and weaned thirteen. Two more litters were born but were killed by the mothers immediately after birth and the number was not ascertained. The nursing period in these animals was greatly prolonged because of the stunted condition of the young. Two females of the second generation were confined to this diet. They grew very poorly and never had any young.

Lot 2183 was fed 9 per cent of protein derived from peas (6 per cent) and muscle (3 per cent). The growth of the rats restricted to this diet was not uniformily good, some reaching the full adult size in the usual period for well fed rats, others remaining permanently undersized. The curves of growth were far superior to any we have ever seen on 9 per cent of pea protein alone (Chart 1, Lot 2376). They were rough coated, old looking, and irritable at 12 months of age.

Two females had each a litter of young (seven young collectively) and destroyed them soon after birth.

Lot 2185 was fed 9 per cent of protein derived from soy beans (6 per cent) and muscle (3 per cent). The growth records were distinctly superior to those we have secured on 9 per cent of soy beans alone (Chart 1, Lot 2510). Their span of life was short. They were very old looking at 14 months.

There was but one female in this group. She had two litters (eight young) but destroyed them soon after they were born.

Chart 11.—Lot 2478 was fed a diet, the protein of which was furnished by barley and liver. It was comparable to those previously discussed in which the protein was derived from cereal or legumes, supplemented with kidney or steak, respectively. The growth and reproduction records of these animals show clearly that liver is superior to muscle as a supplement to barley (compare

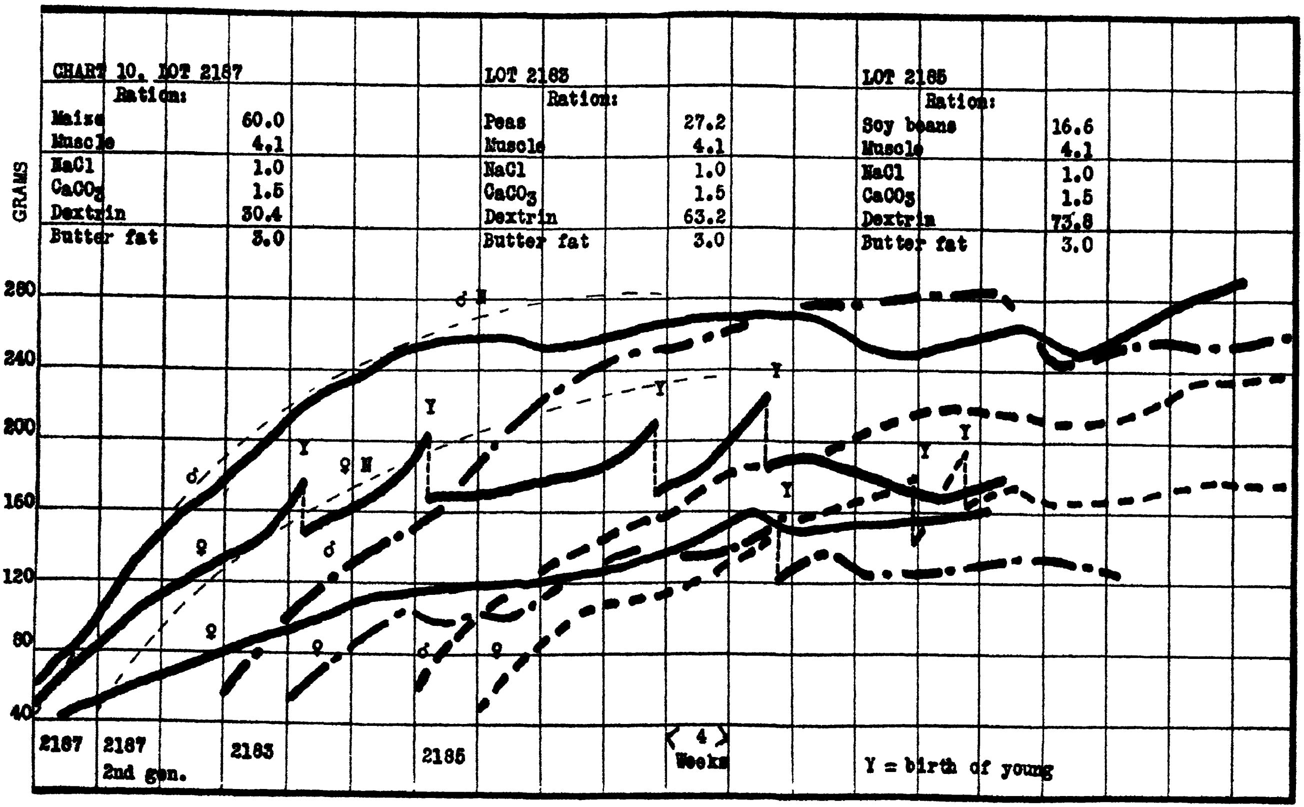
CHART 10. LOT 2187
Ration:
Maize 60.0
Muscle 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 30.4
Butter fat 3.0
LOT 2183
Ration:
Peas 27.2
Muscle 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 63.2
Butter fat 3.0
LOT 2185
Ration:
Soy beans 16.6
Muscle 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 73.8
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
2187
2187 2nd gen.
2183
2185
4 Weeks
Y = birth of young
♂
♀
Y
N

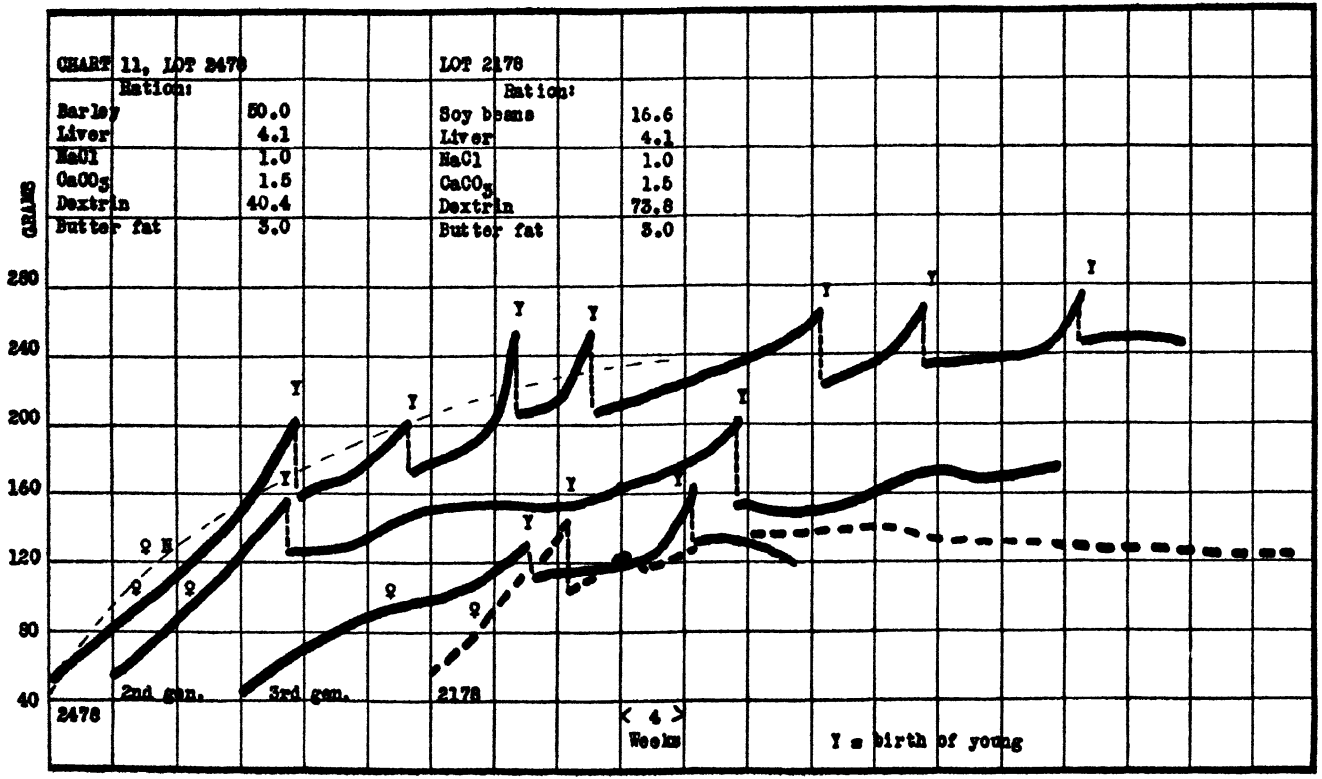
CHART 11, LOT 2478
Ration:
Barley 50.0
Liver 4.1
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 40.4
Butter fat 3.0
LOT 2178
Ration:
Soy beans 16.6
Liver 4.1
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 73.8
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
♀ N
♀
♀
♀
♀
Y
Y
Y
Y
Y
Y
Y
Y
Y
Y
Y
Y
Y
2478
2nd gen.
3rd gen.
2178
< 4 >
Weeks
Y = birth of young

Lot 2477, Chart 9 with Lot 2478, Chart 11). These animals were in good condition at 16 months of age, although their coats were beginning to be rough.

Three females were restricted to the diet. One whose growth was poor was sterile. The other two had collectively fifty-four young (ten litters) and weaned twenty-two. Four second generation females were kept on the diet. Two of these failed to grow in a normal manner and remained sterile. The other two had twenty-six young (four litters) and weaned twenty-five. One third generation female had nine young (two litters) and weaned them all.

Lot 2178 was fed 9 per cent of protein, two-thirds of which was from soy beans and one-third from liver. The growth curves were distinctly below normal, but very much better than could be secured with 9 per cent of soy bean protein alone (Lot 2510, Chart 1). These animals were undersized and old looking at 14 months, which was somewhat earlier than in the case of Lot 2478 fed barley and liver, and distinctly earlier than the time of appearance of senile characteristics in rats fed wheat and liver (Lot 2179, Chart 15).

Two females had collectively eighteen young (three litters) and destroyed them soon after birth.

Chart 12.—Lot 2180 was fed maize and liver proteins as 9 per cent of the food mixture. As in the other diets described in this paper the cereal furnished 6 per cent and the glandular tissue 3 per cent. These growth curves were superior to what could be secured on 9 per cent of maize protein alone, but not so good as were observed in rats fed oats and liver (Lot 2181, Chart 13) or wheat and liver (Lot 2179, Chart 15). These animals were very old looking at 16 months and the young were always undersized and inferior in appearance.

Two females had collectively fifty young (seven litters) and weaned but eight. The cause of the infant mortality in this family was mainly due to inadequate nutrition during the nursing period. This was greatly prolonged because of the stunted condition of the young. Those restricted to the diet after weaning did not thrive. Even in litters which were steadily reduced by the death of individuals at intervals of several days, the survivors did not grow better because of the lessened competition for the mother's milk supply.

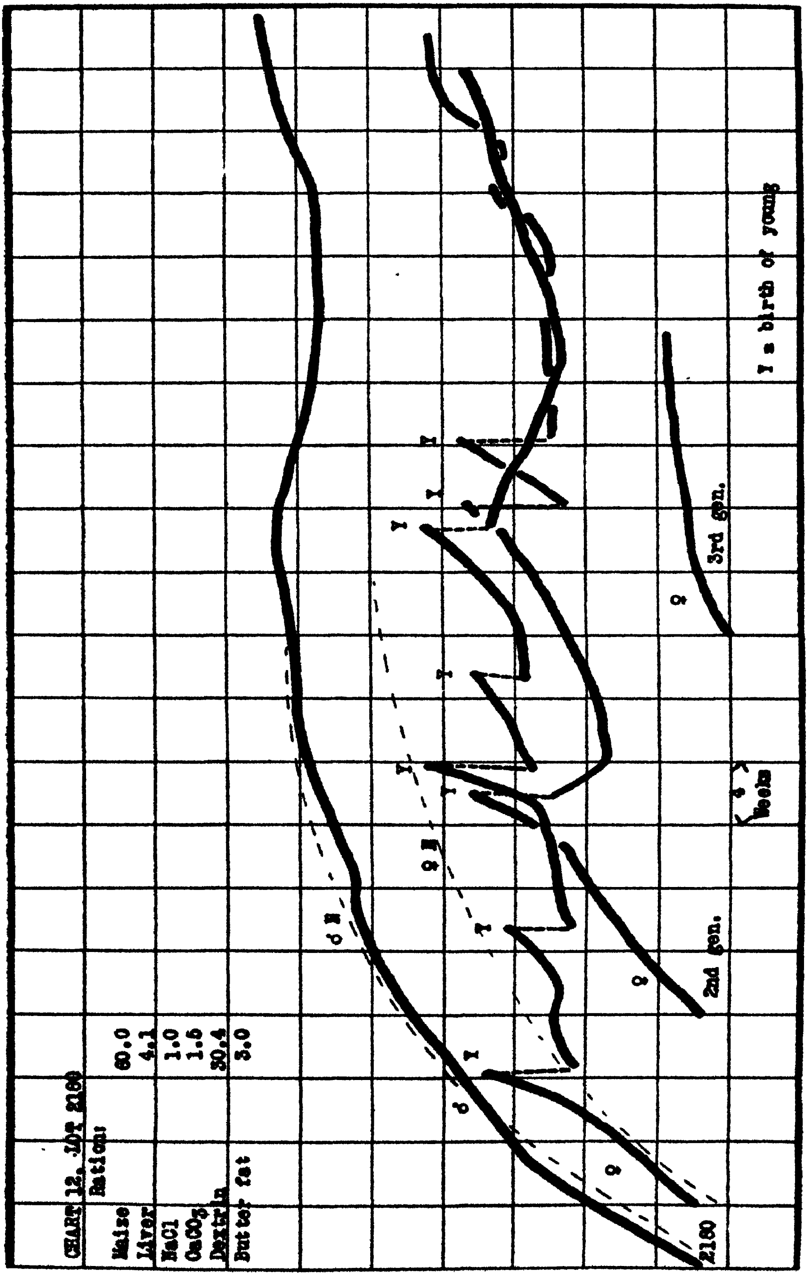
CHART 12. Lot 2160
Ration:
Maize 60.0
Liver 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 30.4
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
2160
♀
♂
♂ M
♀ M
Y
2nd gen.
3rd gen.
4 Weeks
Y = birth of young

Two second generation females grew up on the diet and had collectively twenty-seven young (four litters) and weaned fourteen of them. One third generation female was restricted to the diet but never grew beyond about 75 gm.

A record of infant mortality such as that of this group we know to be the result of poor quality or lack of sufficient quantity of milk secreted by the mother. It seems justifiable to conclude that it was quality rather than the amount of milk which was the determining factor, since the nursing periods were in most cases so prolonged beyond the normal period of 25 days. If the milk flow had been low it would hardly have persisted over so long a period and have enabled a few young to at last reach a state of independence. The young in the second generation were distinctly better once they became able to eat the diet on which the mother was producing milk, than they were while still so immature as to be confined to their mothers' milk as their sole food.

Chart 13.—Lot 2181 was restricted to a diet of rolled oats and liver, the plane of protein intake being 9 per cent of the food mixture. The oats supplied 6 per cent and the liver 3 per cent of the total protein. The inorganic and fat-soluble A deficiencies were made good by suitable additions of salts and butter fat. The growth curves of the group were normal and fertility essentially so, but the mortality of the young was high. The rats in this group began to look old at 14 months and deteriorated rapidly thereafter. The growth curves of this group were far superior to those of Lot 2188 (Chart 9) which grew up on a similar diet with beef muscle in place of liver.

Two females in this group had collectively sixty-eight young (ten litters) and weaned forty. Two second generation females had forty-three young (ten litters) and weaned fourteen. One third generation female had a litter of seven when she had been 6 months on the diet after weaning. She weaned six of these.

All the animals of this group were killed at about 18 months of age, and while they presented shabby exteriors their organs were found at autopsy to be in good condition and there were no lung infections visible.

Chart 14.—Lot 2182 was fed 9 per cent of protein, 6 per cent being furnished by rye and 3 per cent by liver. The growth curves were excellent and the fertility was high. This shows that this

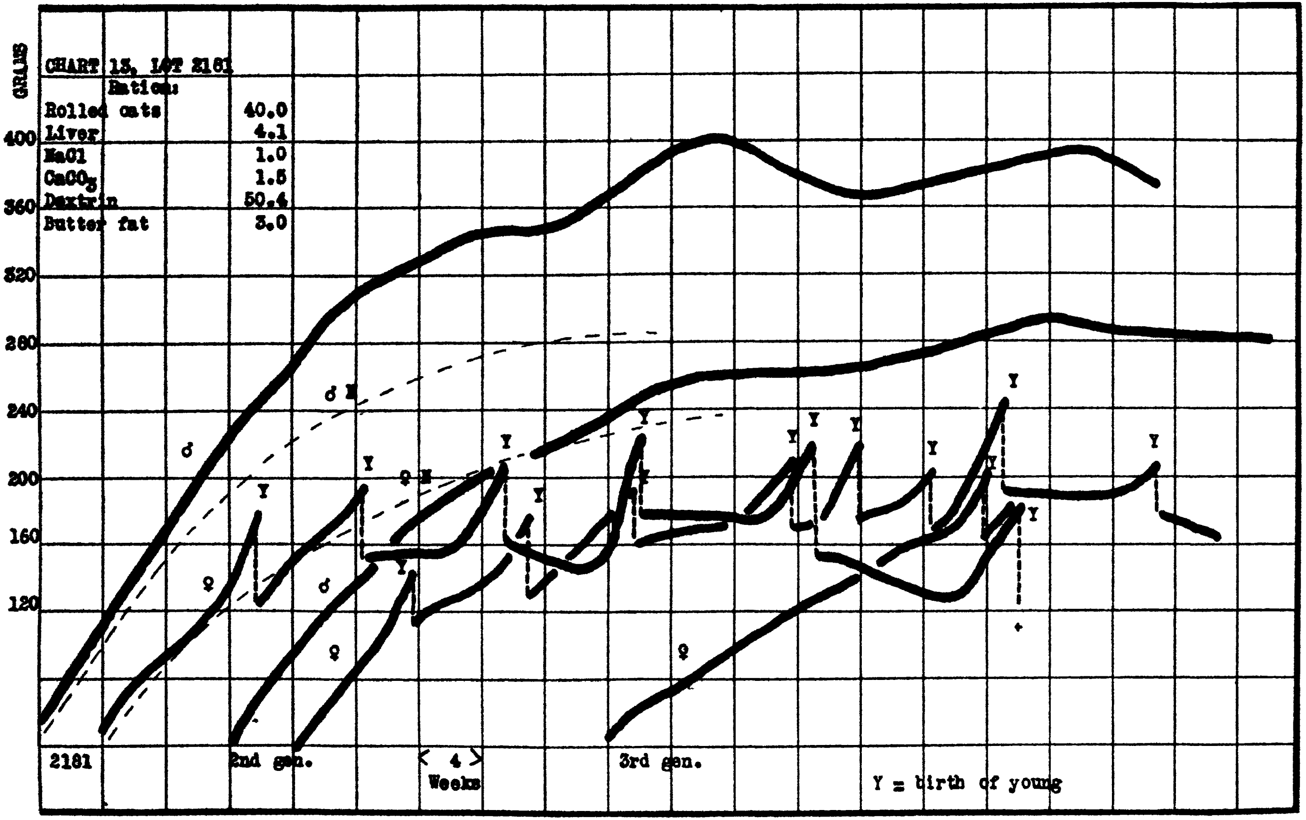
CHART 15, LOT 2181
Ration:
Rolled oats 40.0
Liver 4.1
NaCl 1.0
CaCO3 1.5
Dextrin 50.4
Butter fat 3.0
GRAMS
400
360
320
280
240
200
160
120
♂
♀
♂ II
♀ II
Y
2181
2nd gen.
< 4 >
Weeks
3rd gen.
Y = birth of young

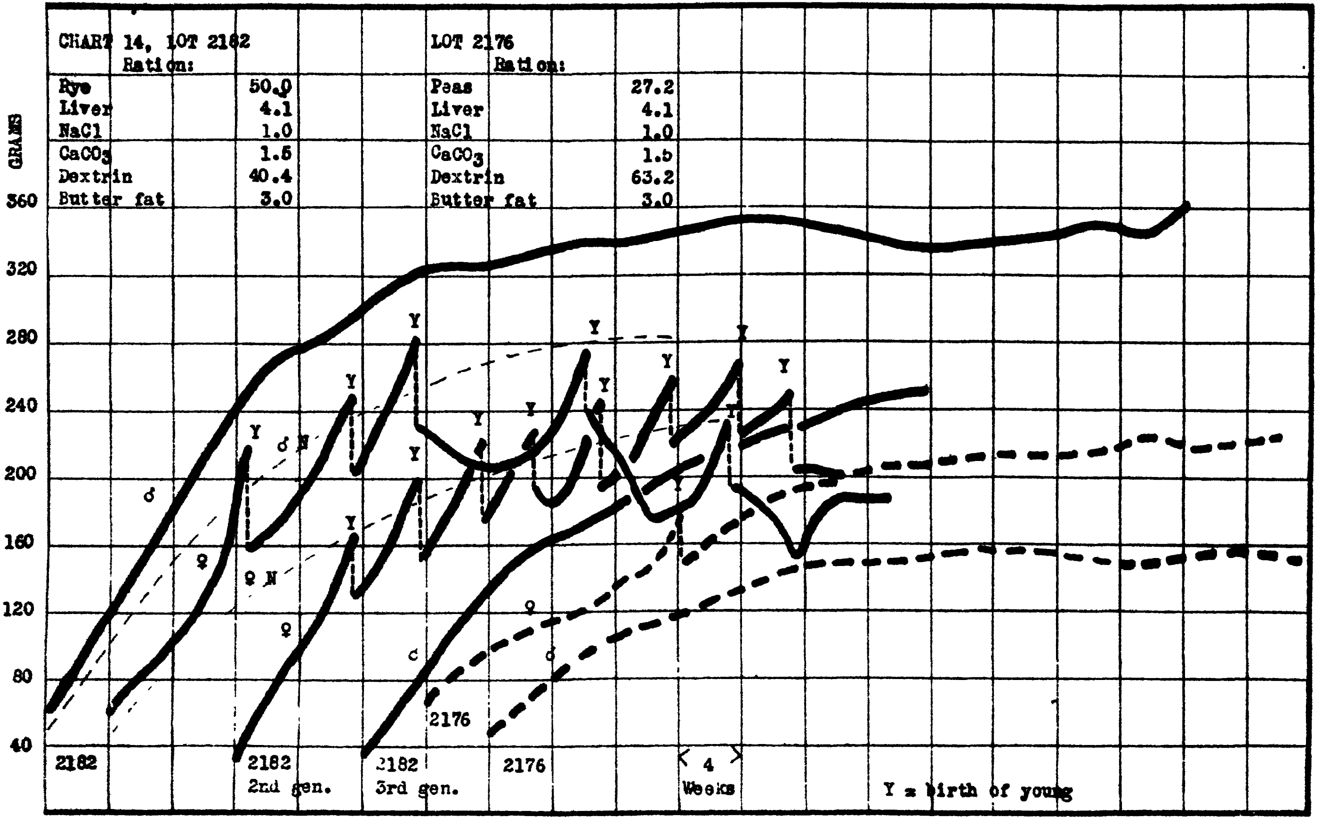
CHART 14, LOT 2182
Ration:
Rye 50.0
Liver 4.1
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 40.4
Butter fat 3.0
LOT 2176
Ration:
Peas 27.2
Liver 4.1
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 63.2
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
Y
♂ N
♀ N
♂
♀
2176
2182
2182
2nd gen.
2182
3rd gen.
2176
< 4 >
Weeks
Y = birth of young

combination of rye and liver proteins is of excellent quality, otherwise 9 per cent of protein would not have sufficed to promote growth so satisfactorily. The infant mortality of this group was, however, very high. This was due to the inadequacy of this amount of protein, even of excellent quality for growth, for the elaboration of milk of suitable quality for the nutrition of the young.

Two females had collectively sixty-one young (nine litters) and weaned thirty-four of them. Three second generation females had fifty young (ten litters) and weaned only eight of them. One of these three had a litter of three and thereafter went 12½ months before having a second litter. One rat (a male) of the third generation was restricted to the diet and grew to a size nearly normal to the adult.

The rats in this group were first observed to show the coat changes characteristic of incipient retrogression toward senility at about 15 to 16 months.

Lot 2176 was fed a 9 per cent diet derived from peas (6 per cent) and liver (3 per cent). The growth curves were poor as compared with any secured with combinations of cereals and liver in comparable amounts. They were undersized and their fertility was low. They assumed the characteristic coat which we have taken as an index to the transition from the youthful to the senile condition at about 13 months.

Three females were restricted to this diet. One had a litter of five young but destroyed them soon after birth. The other two remained sterile.

Chart 15.—Lot 2179 was fed 9 per cent of protein, 6 per cent being derived from wheat and 3 per cent from liver. Their growth curves were normal but they were never in so good a condition as those fed either oats or rye with liver (Lot 2181, Chart 13 and Lot 2182, Chart 14). Their fertility was very high but the mortality of the young was great. They began to look old at 18 months of age.

Two females had collectively 127 young (eighteen litters) and weaned forty-two. Most of these young were destroyed by the mothers soon after birth. The mothers were apparently in good condition and the destruction of the young can be accounted for only on the basis of a peculiar psychological reaction of the mother owing to the nature of the faulty diet.

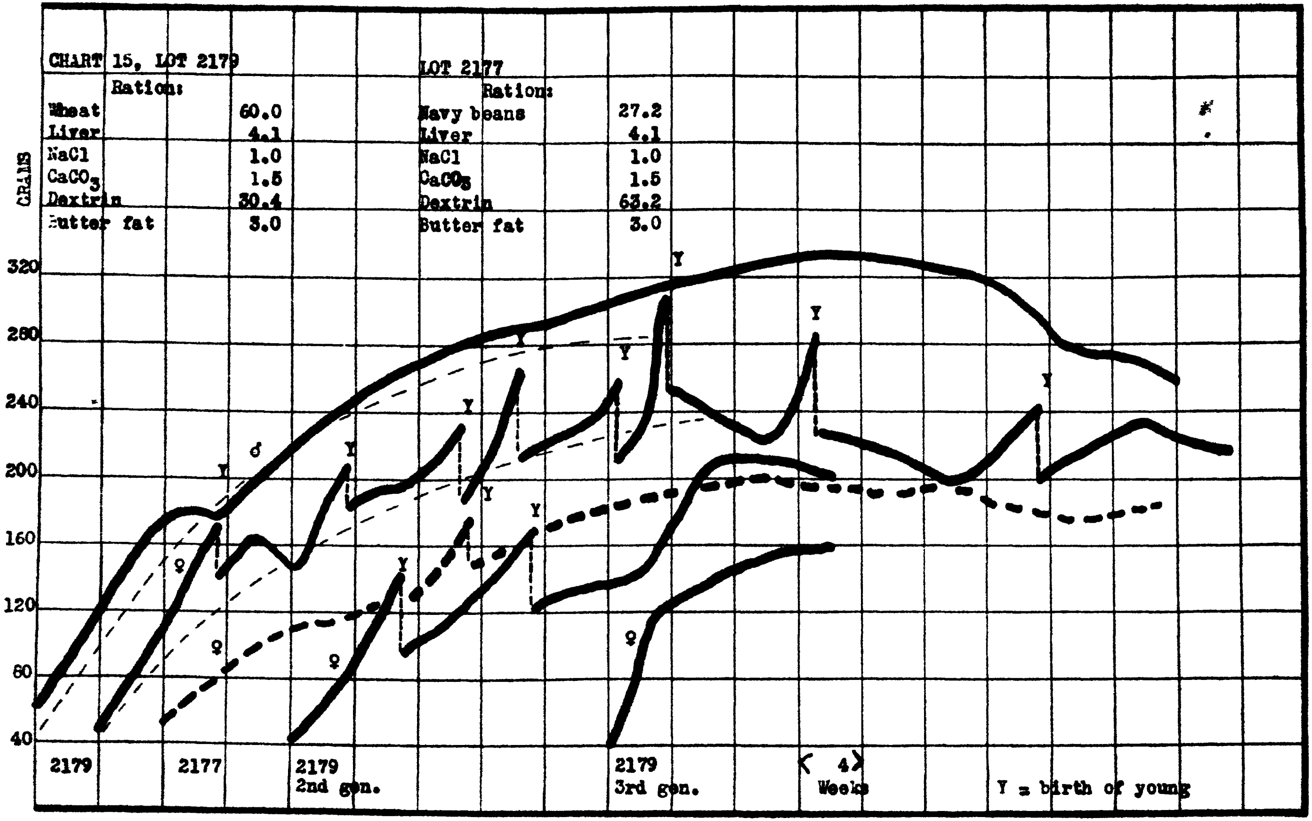
CHART 15, LOT 2179
Ration:
Wheat 60.0
Liver 4.1
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 30.4
Butter fat 3.0
LOT 2177
Ration:
Navy beans 27.2
Liver 4.1
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 63.2
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
Y
♂
♀
2179
2177
2179
2nd gen.
2179
3rd gen.
< 4 >
Weeks
Y = birth of young

Three second generation females had collectively twenty-three young (four litters) and weaned only one. This sole survivor was kept on the diet for 3½ months but was inferior in development and was discarded.

Diets of this type in which the protein intake was about 18 per cent, and in which 11 per cent was furnished by cereals and legume seeds and 7 per cent by liver, kidney, or muscle, have proved excellent for promoting fertility and rearing of young with very low mortality. This proves conclusively that in the series of experiments reported in this paper the limiting factor in growth, fertility, and success in rearing young was protein.

Lot 2177 was fed navy beans to furnish 6 per cent of protein and liver to furnish 3 per cent. The growth curves were inferior to any observed on combinations of a cereal and liver, but comparable to those of rats fed peas and liver in comparable amounts (Lot 2176, Chart 14). Growth was, however, far superior to any secured with 9 per cent of navy bean protein alone (Lot 2368, Chart 1).

These animals appeared *very old* at 15 months. There were three females in the group. One had a litter of two which she destroyed soon after birth. The other two remained sterile.

Chart 16.—Lot 2392 was fed a diet containing but 4 per cent of protein derived from potato (1 per cent) and liver (3 per cent). A very small amount of nitrogen in this and the following rations was added in the alcoholic extract of wheat germ. The rats were unable to grow on this diet, all factors in which, other than protein, were of fairly satisfactory quality. In Period 2 purified protein (casein) was added. This modification of the diet was followed by a sharp response to growth.

Lot 2394 was fed a diet identical with that of Lot 2392 except that muscle tissue (round steak) replaced the liver. The animals grew very slowly on this food mixture containing but 4 per cent of protein. In Period 2 the protein moiety of the diet was enhanced by the addition of casein. This led to growth at a rapid rate.

Lot 2393 was fed a diet identical with those of Lots 2392 and 2394 except that kidney replaced the liver and muscle, respectively. This diet also contained 4 per cent of protein. Growth at a slow rate was possible on this diet. In Period 2 casein was added and this led to a stimulation of growth. It will be seen from the curves

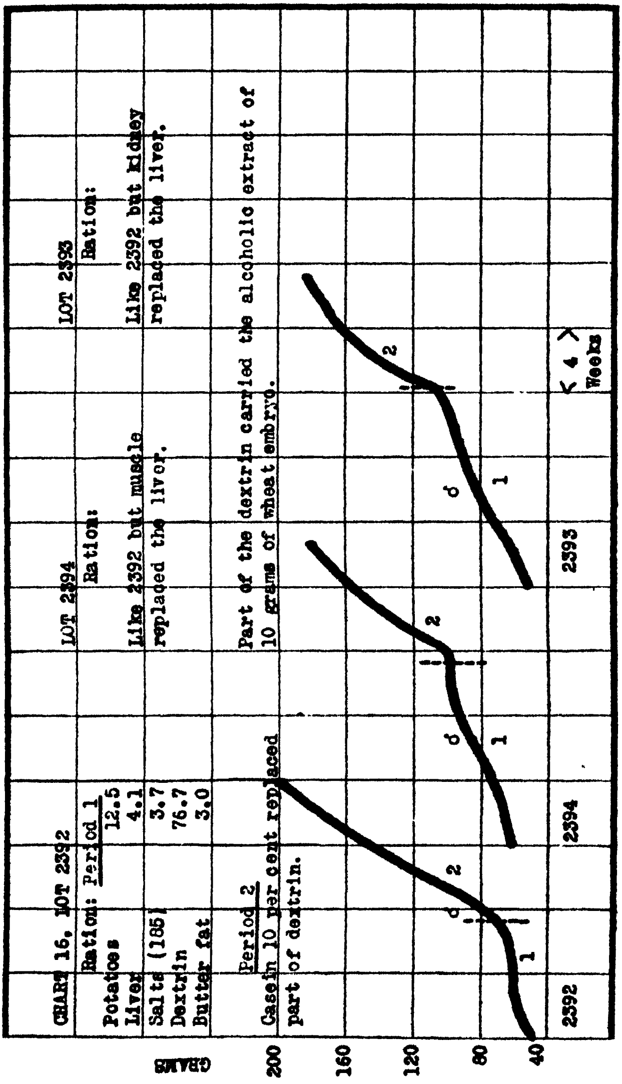
CHART 16. LOT 2392
Ration: Period 1
Potatoes 12.5
Liver 4.1
Salts (185) 3.7
Dextrin 76.7
Butter fat 3.0
Period 2
Casein 10 per cent replaced part of dextrin.
LOT 2394
Ration:
Like 2392 but muscle replaced the liver.
Part of the dextrin carried the alcoholic extract of 10 grams of wheat embryo.
LOT 2393
Ration:
Like 2392 but kidney replaced the liver.
GRAMS
200
160
120
80
40
2392
2394
2393
< 4 >
Weeks
♂
1
2
♂
1
2
♂
1
2

presented in the chart, each of which is representative of a group of four animals, that kidney protein supplements the nitrogen of the potato better than either liver or muscle proteins. These curves are included in this series to illustrate the fact that certain combinations of proteins are of sufficiently good quality to enable young rats to grow nearly as well on diets containing but 4 per cent of protein (potato and kidney) as they do on rations of similar value with respect to non-protein factors in which the sole source of nitrogen is 9 per cent of legume proteins (pea, navy bean, and soy bean). Compare Chart 1, Lots 2376, 2368, and 2510 with Chart 16.

SUPPLEMENTARY PROTEIN VALUES IN FOODS.

III. THE SUPPLEMENTARY DIETARY RELATIONS BETWEEN THE PROTEINS OF THE CEREAL GRAINS AND THE POTATO.

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It has become well established that the diet of man or animals may, when defective in sufficient degree, produce at least two distinct syndromes, scurvy and beri-beri. Experimental studies on animals leave no room for doubt that a third specific deficiency disease, xerophthalmia or keratomalacia, is produced by lack of a definite organic factor essential for normal nutrition. There is much reason to suspect that certain eye conditions popularly referred to as night-blindness, and in the literature as hemeralopia may be, in part at least, due to lack of sufficient fat-soluble A. The widespread infections of the eyes seen in Egypt and elsewhere, may have their origin in lowered vitality of the eye structures due to lack of the dietary factor, fat-soluble A, which favors the invasion of the eyes by microorganisms. The evidence seems sufficient to warrant the conclusion that there are three deficiency diseases which occur in man as well as in animals.

It is natural that the first step toward an appreciation of the relation of the diet to health in man should have been the result of casual observation on the extreme conditions, scurvy and beri-beri. The rarity of reference to the relation of faulty diet to the general health of peoples in the writings of those who are now discussing health problems, shows that it is not at present accepted by many that there is any important relation of the food supply to well being, except in extreme cases of one-sided diet in which the defects are sufficiently grave to induce the development of a "deficiency" disease. The reason for this is easy to understand. Our standards of what constitutes wellness and illness, as well as of what constitutes normal physical development, are relatively low. People are generally considered well until they admit that they are ill, or until they look so unwell as to attract attention. The difference between the average physical development of men and women, as contrasted with the best specimens in the community, is very great, yet we are accustomed to regard as normal many persons who are but poorly constituted physically.

Malnutrition is painless, and leads to susceptibility to other agencies which may induce discomfort. The sequence of events is not ordinarily appreciated, and the cause, therefore, escapes notice. Low vitality, low resistance, and inefficiency, and a tendency to cumulative fatigue, are what we should expect in man to result from adherence to a faulty diet when the faults are of a lower order than would be necessary to bring about an attack of a "deficiency" disease. Although a few leaders in the new movement for the betterment of the health condition of children are urging the importance of properly selected food, the idea is new and not generally appreciated. There is much reason to believe that one of the most important contributing factors in industrial fatigue is the low recuperative power that results from an unsatisfactorily selected food supply. The data regarding the animals described in this paper, all of which were subjected to diets which were faulty to a certain extent, afford striking evidence that such types of diets interfere with the completion of the life history in a normal manner. This is true, notwithstanding the fact that in some instances the faults were by no means marked. The extent to which the animals were injured, and the manner in which they were affected, afford food for serious reflection concerning the similar experience of man and its probable causes.

The results of our experimental studies on the rat have led us to an appreciation of the short-sightedness of the view that a diet is satisfactory if it is not sufficiently poor to cause the development of a "deficiency" disease. Although the importance of a diet containing an abundance of milk and eggs has become generally accepted to have a distinct therapeutic value in the treatment of tuberculosis, it has not been widely appreciated that a similar diet should be very effective as a preventive of infection or of the flaring up of the disease.

We have given a great deal of attention to the problem of demonstrating the effects of relatively slight defects in the diet on the general health of the rat, and on its capacity to reproduce and rear young, and to remain vigorous to an advanced age. We have united the study of these problems with the study of the extent to which the proteins of certain foods supplement those of others. It has been easily possible to develop these lines together, and indeed it was not possible to pursue the one phase of the work independently of the other, for in such systematic studies one meets with the nice gradations in quality of proteins which afford the opportunity to make refined observations on the physiological well being of the animals as affected by the variations in the diet.

We deem it a matter of great importance to discover in its incipiency when an animal is brought into a state of nutritive instability. Under such conditions it is unable to support a burden of any appreciable magnitude, and it matters little whether the burden be that of producing milk, or bearing young, or of withstanding fatigue. It is such shades of difference in quality which occur in human experience in numerous cases. The type of experiment which we have come to employ, in which the performance of reproduction and nursing of young comes under observation, brings to light this "twilight zone" of nutrition in which the vital powers of the body are below the optimum.

It is desirable at this time to discuss the changes in appearance of the rat, and in its behavior as it grows old, in order to make possible an appreciation of the manner in which we have managed our experiments.

The well fed rat exhibits up to about 18 months of age the appearance of full vigor as shown by plumpness of the muscles; bright, prominent eyes; clean, smooth, pink skin, easily observable on the feet and ears. The tail appears clean and free from scales. The coat appears to consist of hairs of approximately uniform length, and is glossy and well kept. Such animals are not apprehensive and permit themselves to be handled without becoming annoyed. They do not attempt to escape when handled with care.

At a certain point in their life history there is a characteristic change in the appearance of the coat. This consists of the prominence of certain hairs which are scattered over the entire body, and which look coarse and are distinctly longer than the under coat.

Physiologically youthful rats, irrespective of age, give attention to their coats. When the latter differentiates into hairs of two sorts the animals become neglectful of their toilets and look less clean and attractive than formerly. The skin on the ears, feet, and tail generally loses its waxy and healthy surface and appears dry, harsh, and often scaly.

With this change in appearance there is usually seen a change in the habits of the animals. They tend to sit and sleep with the head tucked under the body or with the face downward. Healthy youthful rats sleep lying stretched out on their ventral surface or partly turned on one side, with the front legs extended and with the head resting on the paws. When the cage of youthful rats is opened they are awakened if asleep, and show an interest in their observer, but do not show signs of fear. Animals which are developing senile characters are generally dozing and their attention is not usually attracted by careful opening of the cage. When touched they spring up in surprise and show irritation at being molested. As time passes this behavior becomes more marked. The irritability, somnolence, dirtiness, roughness of the coat, and scantiness of hair become progressively more noticeable. To some extent, according to the nature of the fault in the diet, specific changes appear, such as attenuation of form and baldness over limited areas, but these latter are more characteristic of animals fed certain foods than they are of diets from different sources having like specific dietary deficiencies. A further example of this peculiar effect of diet due to some obscure causes is seen in the frequent occurrence of very fine, short, glossy hair, suggestive of a mole in rats fed largely on maize or kafir corn.[1]

Another type of observation which has proved to be of value as an index to the quality of the diet which enables one to make fine shades of distinction in the value of the protein factor in a series of experiments which are properly planned, is the success of the females in rearing their young. This is a more sensitive index than is fertility, although in general low fertility and high infant mortality tend to run parellel. The fertility may, however, be high, and but a small number of young be reared to the weaning age. This mortality is, we believe, generally due to poor quality of the milk secreted by the mothers rather than lack of milk production. It is not always possible to get satisfactory information on this point, but since the stomach when filled with milk can be readily seen in a young rat during the first few days when the animal is properly illuminated, we have been able to observe the amount of milk produced in a sufficient number of cases to lead us to believe that in general the failure of the young to grow is due to deficiencies in the composition of the milk rather than to lack of sufficient amount.

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[1] McCollum, E. V., and Simmonds, N., Unpublished data.

There is a marked tendency among female rats fed such diets as are described in this paper to destroy their new-born young. If for any reason they become scattered about the cage the mothers will not be solicitous about returning them. This abnormal psychological reaction of the mothers toward their young represents a new and hitherto unstudied effect of faulty nutrition. It serves to emphasize the deep seated relation of the character of the diet to the mental reactions generally, for if the maternal instinct is so perverted, those attributes of the nervous system which are less deeply ingrained should be influenced in even greater degree.

In contrast to the behavior of the mothers on experimental diets, details of which are given in the legends to the charts, it is of interest to contrast the behavior of the mothers in our breeding stock. It is our custom to isolate the females when it is easily observable that they are pregnant. This cannot be determined with certainty until about the end of the second week after fertilization. For some years it has been our custom to isolate two or three females in our breeding stock in a single cage. They each prepare a nest for the reception of their young, but when the cage is cleaned within a few days, and fresh bedding replaces the old, the young are mixed and returned to the cage together. From this time on the mothers do not separate their own young, but allow them to remain together in one nest, which may contain as many as twenty to thirty individuals. They nurse each other's young indiscriminately. A mother will spread herself over the nurslings, and as many as possible attach themselves to her. After a time she will free herself from them and another mother will take her place. They seldom kill a young one and the infant mortality is almost zero. Only very infrequently have we seen a young rat under such circumstances unable to compete successfully with the others for his share of the milk supply.

In experimental animals on diets which are faulty, it appears, as stated above, that the milk flow is ordinarily sufficient to meet the needs of the developing young, but the quality may be poor with respect to some one or more factors, and cause failure of the animals to grow satisfactorily. It appears, however, that some mothers do not produce enough milk, at least after a time: We have occasionally seen a litter grow fairly well for 4 to 10 days, then all die within a few days. The cause of such behavior has in general not been ascertained.

We have given some attention to determining how long a female rat may continue to give milk provided she is kept with young of a suitable size. In one case in which the young were removed and replaced by smaller ones at intervals of a few days, a female rat continued to secrete milk up to 141 days in quantity sufficient to induce some growth in four young about 8 to 10 days old. It is known that these young did not eat of the mother's food supply.[1]

When the fault or faults in a diet are sufficiently marked, growth is interfered with in the young, and the effects soon become apparent in the external appearance of the animals, even in the adult. We have made an effort to determine *how sensitive* the rat is to slight degrees of faulty composition in its diet. This can be done by prolonging the period of observation far beyond the growth period, and including a record of the fertility and success in rearing young and the time of the appearance of the first signs of old age. Since the character of the diet is but one of the factors which influence the well being of an animal, we have maintained the hygienic conditions such as illumination, temperature, ventilation, and opportunity for exercise uniform throughout our entire rat colony. Furthermore, the differences in the condition and vitality of the animals as measured by the tests described are due in all cases to variation in the quality of the diet, which falls within the limits of what may occur in combinations of ordinary natural foods of animal and vegetable origin.

As an example of the objective which we have had in mind in conducting these studies, the comparison of the relative values of the proteins in the several rations may be cited. The value of the proteins in a food or mixture, or the value of a purified protein, has not been studied by methods more sensitive than that of comparing their efficiency for the support of growth. We have learned that much more accurate comparison of quality in proteins can be made by maintaining a series of animals on a diet otherwise comparable, but containing various levels of the same source of protein throughout the growing period and into adult life, so as to discover the plane which just suffices for this result without inducing inferiority in appearance or size. Under these conditions the mother rat should she produce and attempt to suckle a litter would be confronted with an added demand for nutriment by the young. The young at the nursing age are more sensitive to faulty diets than are older animals which have been satisfactorily nourished during the first weeks of life. We have thus set up experimental conditions in which an unusual demand is made upon the mother, and extraordinary sensitiveness is characteristic of the young because of their early age and lack of reserve power.

It is of more than ordinary interest to find, as we have, that a diet may be sufficiently good to enable a group of animals to grow to apparently the normal adult size, and even to support a fair amount of reproduction and moderate success in the nursing of young to a state of independence, and yet be inadequate for the nourishment of a family through several generations. This fact is brought out well in many of our studies in which families during several generations have been restricted to a monotonous diet. If the diet is properly constituted with respect to all its parts the animals tolerate, at least to the fifth generation, such a monotonous food supply without any signs of injury. If, however, there are factors which are even slightly below the optimum, the vitality of the animals falls off in each succeeding generation, and the strain dies out. Sometimes this happens in the second, third, or fourth generation.

An interesting difference in the effect of stunting of young animals is seen, depending on the cause of the interference with growth. When the protein content of the ration is too low, young rats remain approximately normal in form, or at least they remain long and lithe. When the inorganic content of the ration is faulty on the other hand, they develop a short and stocky form. In the former case they respond to a better protein supply and look approximately normal, but when deformed by lack of sufficient calcium they never assume the normal form as the result of correction of the diet.[1]

The discussion of the growth, fertility, infant mortality, and appearance of the first recognizable signs of senility which is presented in the legends to the charts, will make clear the fact that even very slight differences in the quality of the food mixture with respect to protein and fat-soluble A at least, can exercise a marked influence on the vitality of the rat. Animals may be brought to a state by feeding ordinary cereals and potato, supplemented in a fairly satisfactory manner except for the protein factor, in which their nutritive condition is unstable, and where very slight shifts in the character of the food for better or worse, make easily observable differences in their capacity to perform satisfactorily the functions of reproduction, or to retain full vigor to an advanced age.

The experimental work reported in this paper is limited to a study of diets in which the protein was derived from a mixture of a cereal grain and potato. The quality of protein was estimated as accurately as possible by existing methods. In some cases casein was added to the diet in order to bring out the fact that the protein in a certain mixture was actually the limiting factor. The results show that the potato nitrogen serves to some extent to supplement that of the cereal grains, but that it is not so valuable for this purpose as are certain animal tissues such as kidney, liver, or muscle.[2]

The data furnish definite evidence that there is no marked difference in the capacity of the nitrogen compounds of the potato to enhance those of the different cereal grains, as was found to be the case in similar combinations of cereals with animal tissues. The latter are individually more effective with certain grains than with others, whereas the potato is less highly valuable in any combination, and more similar in its relations to each.

These studies lend support to the general proposition that it is not wise to attempt to combine foods which have the same function, such as have the seeds and tubers. Both are storage tissues of plants and have in great measure the same dietary properties. They do not, therefore, serve effectively to make good each other's shortcomings.

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[2] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 139.

SUMMARY.

We have described methods of experimenting which are more sensitive than rate of growth for yielding information concerning slight gradations of quality in foods. These involve fertility, success with the rearing of young, longevity, the preservation of youthful characters, and the stability of the nervous system. By establishing standards for comparison these indexes to well being reveal with great refinement the physiological condition of the animal.

The supplementary values of the nitrogen compounds of the potato for those of the cereal grains and legume seeds have been studied with a view to making refined observations as to the biological values of several combinations. These experiments have brought to light the following facts:

The nitrogenous compounds of the potato tend in some degree to enhance the biological value of proteins of cereals and legume seeds, but not to as great an extent as do the proteins of such animal products as kidney, liver, muscle,[2] or milk.

Marked differences are observed in the extent to which animal tissues enhance the quality of the proteins of individual seeds. No such selective efficiency has been observed in the relation of the amino-acids of the potato to those of cereal or legume seeds. The potato seems about equally effective as a supplement for all which have been studied.

Chart 1.—These records are typical growth curves of rats fed diets in which wheat, rolled oats, maize, peas, and millet seed, respectively, furnish the only source of protein. The amount of each seed was adjusted so as to make the protein content of the diets 9 per cent of the food mixture. In each case the necessary inorganic salts and butter fat were added to make good the well established deficiencies of these seeds.

Wheat proteins are seen to produce the best growth curves and the highest fertility. No young were secured from the animals fed maize, peas, or millet seed. A few young were reared on the wheat and oat diets. Charts 5 and 8 show that the infant mortality is in a great measure due to inadequate protein moiety of these diets. These records serve as the basis of comparison with those contained in the other charts.

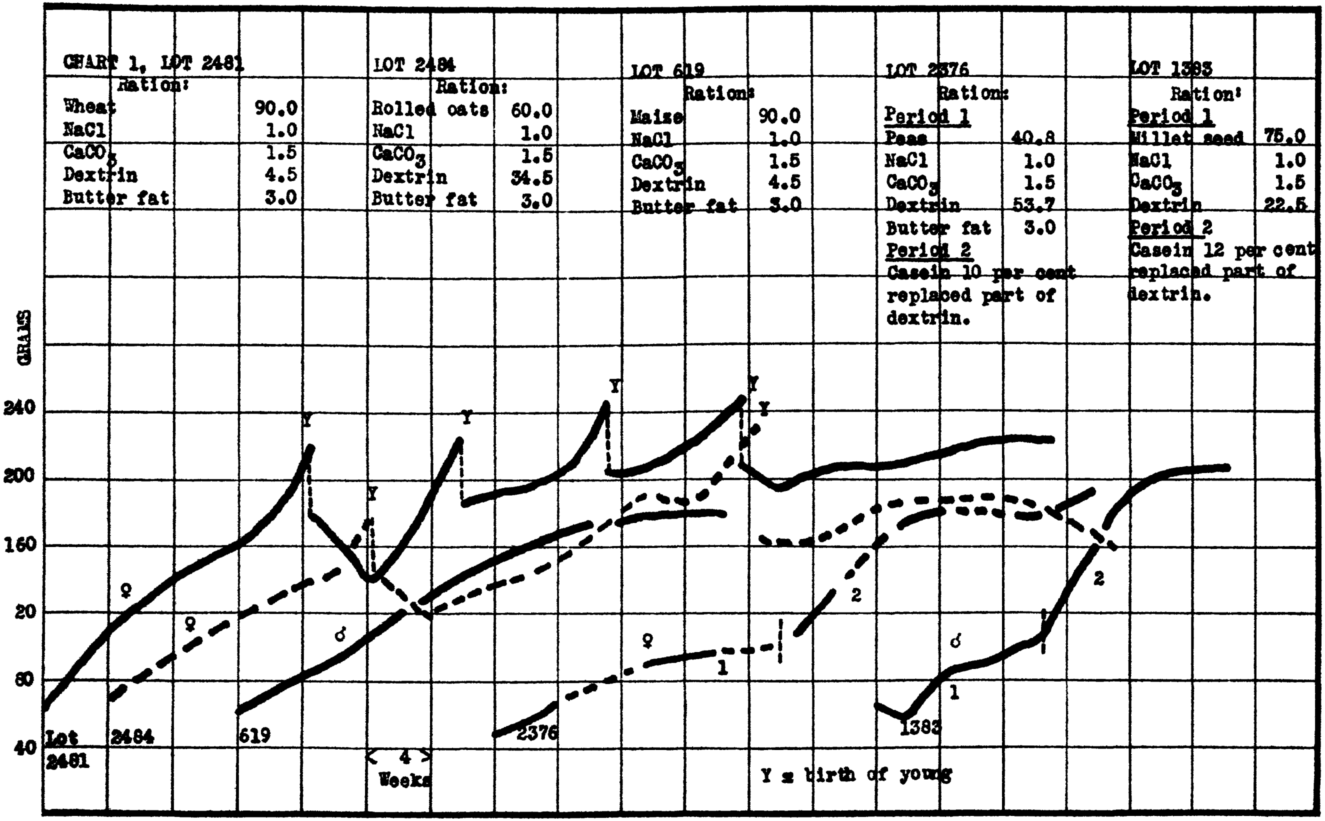
CHART 1, LOT 2481
Ration:
Wheat 90.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 4.5
Butter fat 3.0
LOT 2484
Ration:
Rolled oats 60.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 34.5
Butter fat 3.0
LOT 619
Ration:
Maize 90.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 4.5
Butter fat 3.0
LOT 2376
Ration:
Period 1
Peas 40.8
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 53.7
Butter fat 3.0
Period 2
Casein 10 per cent replaced part of dextrin.
LOT 1383
Ration:
Period 1
Millet seed 75.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 22.5
Period 2
Casein 12 per cent replaced part of dextrin.
GRAMS
240
200
160
20
80
40
Lot 2481
2484
619
2376
1383
♀
♀
♂
♀
♂
1
2
1
2
Y
Y
Y
Y
Y
Y
< 4 >
Weeks
Y = birth of young

In all cases the food mixture was ground together so that all its ingredients were consumed in the proportions shown in the formula, as is our invariable practice.

Chart 2.—These curves illustrate the growth and reproduction of a group of rats fed from weaning time on diets, the protein of which was derived from millet seed and potato. This seed and tuber served as the only source of fat-soluble A.

Lot 1398 contained about 11 per cent of protein (N × 6.25), approximately 9 per cent being derived from millet seed and 2 per cent from potato. The growth records were poor, but distinctly better than was observed in Lot 1383 (Chart 1), in which 9 per cent of protein derived entirely from millet seed was the sole source of nitrogen. No young were secured in the latter group, whereas a few were secured from Lot 1398. This group contained two females. These had together eighteen young (three litters) of which nine were weaned. Two of their daughters were reared on the diet but remained sterile. The young of this group were all undersized for their age and the nursing period had to be prolonged to approximately twice the normal period of 25 days in order to enable the young to survive on the family diet. There is every reason to believe that the high mortality of the young in this group was the result of qualitative inadequacy of the milk secreted by the mothers. All these rats aged early. They were very senile at 8 to 10 months.

Lot 1452 had a diet like that of Lot 1398 except that it contained 10 per cent of casein to supplement the protein. The good effects of this addition are easily seen in the character of the growth curves. Millet seed does not appear to be a very wholesome food grain. The fertility of the females in this group was far below normal and the mortality of the young high. These young were not destroyed soon after birth but only after they had time to show from retarded growth that the quality of the milk of the mother was inferior.

Two females had together twenty-six young (three litters) of which twenty were weaned. Two daughters were confined to this diet. One had one litter of four young which she destroyed. The other had one litter which she destroyed, and died in the delivery of a second litter.

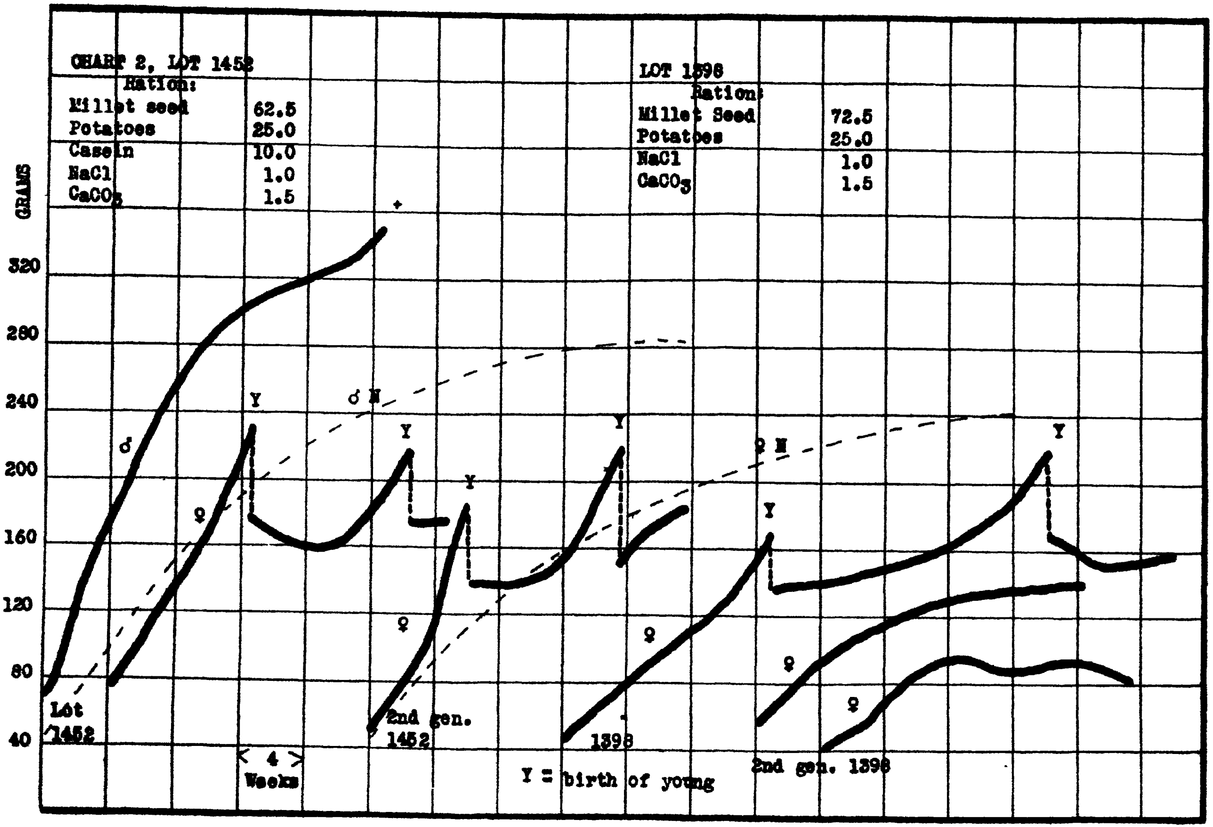
CHART 2, LOT 1452
Ration:
Millet seed 62.5
Potatoes 25.0
Casein 10.0
NaCl 1.0
CaCO$_3$ 1.5
LOT 1398
Ration:
Millet Seed 72.5
Potatoes 25.0
NaCl 1.0
CaCO$_3$ 1.5
GRAMS
320
280
240
200
160
120
80
40
♂
♀
Y
♂ N
♀ N
Lot 1452
2nd gen. 1452
1398
2nd gen. 1398
< 4 > Weeks
Y = birth of young

Chart 3.—The animals in Lots 1407 and 1461 were fed diets similar to those of Lots 1452 and 1398, respectively (Chart 2), except that the former had an added source of fat-soluble A. Since millet seed contains distinctly more of this factor than wheat or oats, or than peas or beans, the effects of adding butter fat to this ration was scarcely noticeable.

Lot 1407 contained three females. They had altogether thirty-four young (five litters) and weaned fourteen of them. Two females of the second generation were confined to this diet. One had a litter of six but they were very puny. Some of these were destroyed when very young. A litter which was nursed 11 days was then only about 60 per cent as heavy as it should have been if the mother's diet had been satisfactory.

Lot 1461 was fed millet seed, potatoes, butter fat, and casein in Period 1. Little growth was possible on this food. In Period 2 sodium chloride and calcium carbonate were added. Growth took place at a rapid rate after this addition was made. There were three females in this group. They had forty-six young (seven litters) of which thirty-one were weaned. One female of the second generation was restricted to the diet and had a litter of eleven young but killed them all, and died herself soon afterward. Other young from the original group on this diet were very inferior. Where these young were not destroyed when very small they remained stunted while nursing. This we interpret as being due to faulty composition of the milk secreted by the mother. It is probable this was the result of some toxic substance passing into the milk from the millet seed.

These records all show that millet seed when fed in liberal amounts causes injury. This we provisionally interpret as due to the presence of some toxic substance in millet seed. That a shortage of fat-soluble A was not responsible for the results observed is shown in Chart 2. Among the farmers of the middle west there is a common belief that liberal feeding of millet causes injury to farm animals. The toxicity of millet seed is still under investigation.

Chart 4.—The records in this chart are those of rats which were fed diets in which wheat and potatoes furnished the sole source of fat-soluble A. The protein content in the diet of Lot 1399 was approximately 9 per cent. That this amount of protein from these

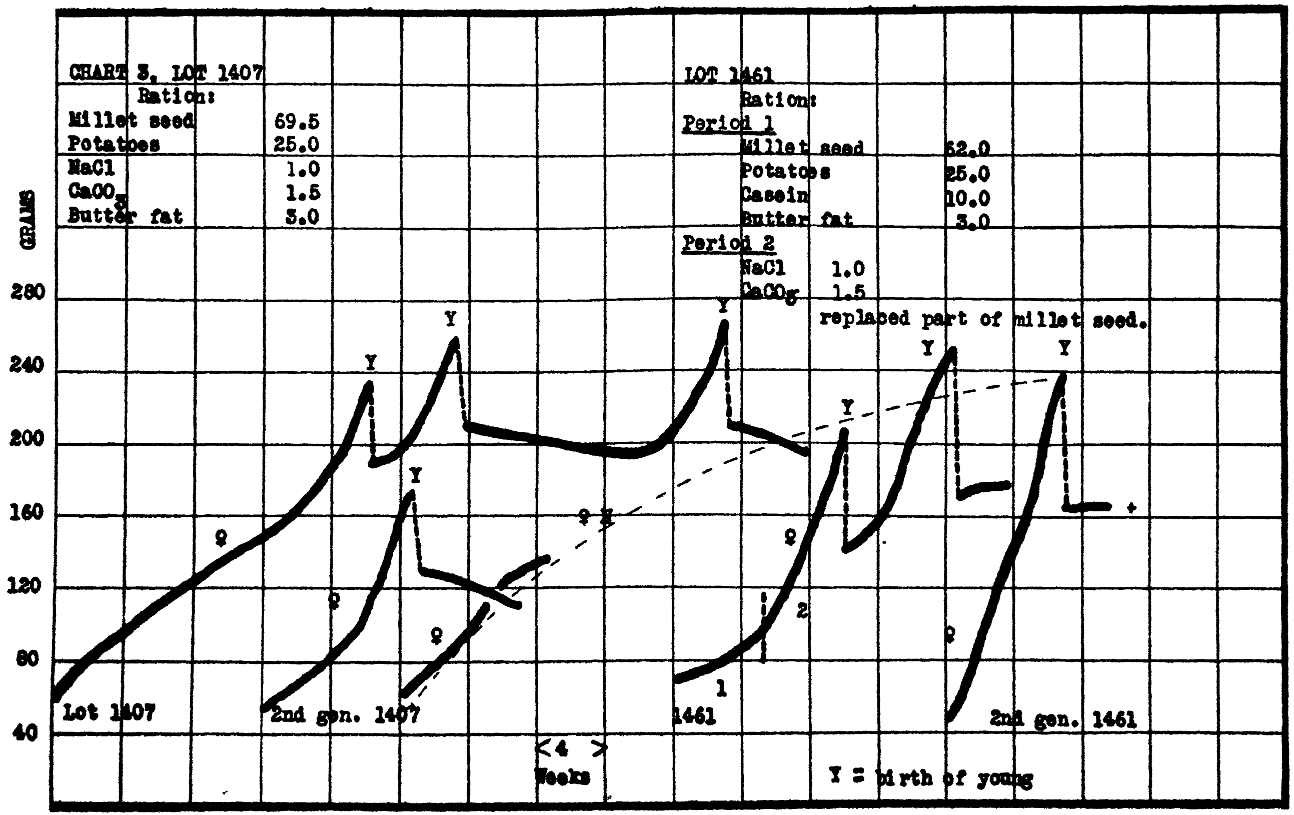
CHART 3, LOT 1407
Ration:
Millet seed 69.5
Potatoes 25.0
NaCl 1.0
$CaCO_3$ 1.5
Butter fat 3.0
LOT 1461
Ration:
Period 1
Millet seed 62.0
Potatoes 25.0
Casein 10.0
Butter fat 3.0
Period 2
NaCl 1.0
$CaCO_3$ 1.5
replaced part of millet seed.
GRAMS
280
240
200
160
120
80
40
Y
Y
Y
Y
Y
Y
Y
Y
♀
♀
♀
♀ ♂
♀
♀
1
2
Lot 1407
2nd gen. 1407
1461
2nd gen. 1461
< 4 >
Weeks
Y = birth of young
+

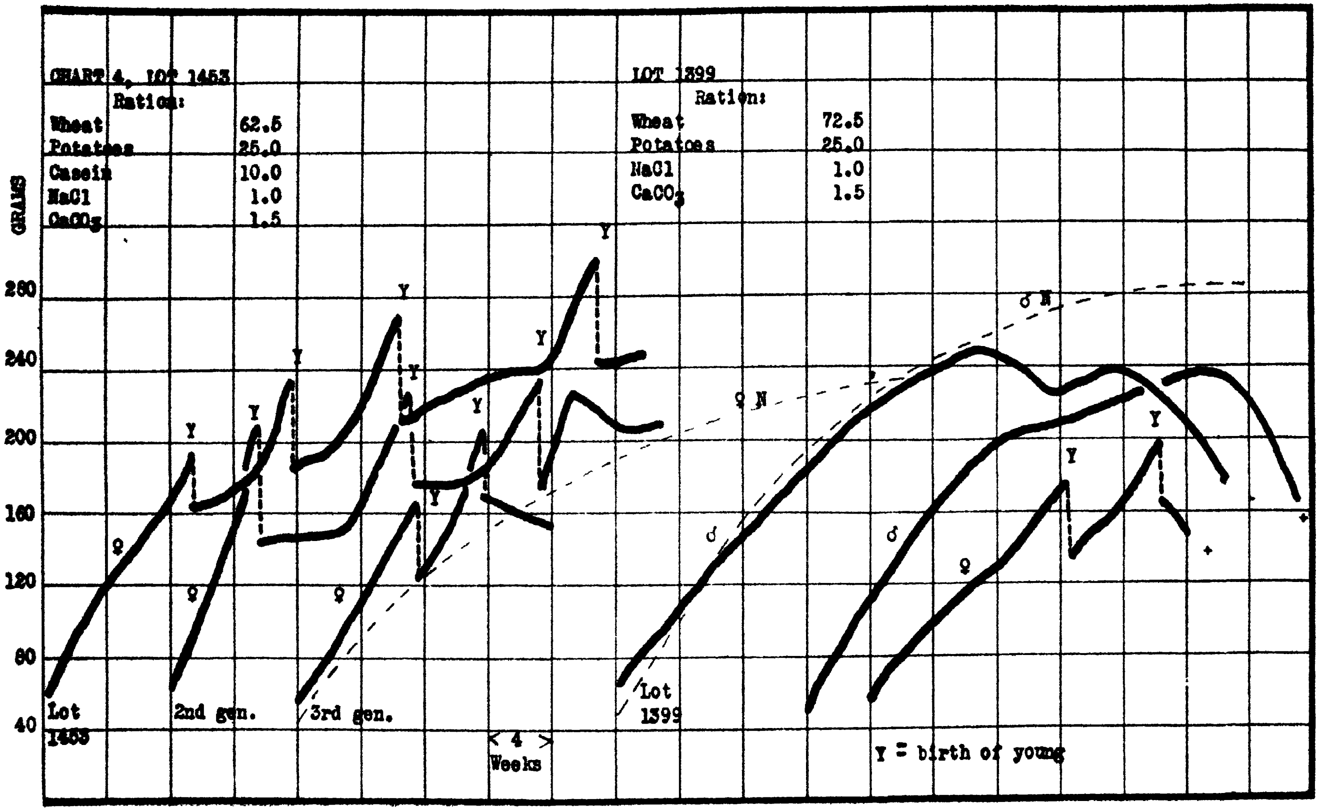
CHART 4, LOT 1453
Ration:
Wheat 62.5
Potatoes 25.0
Casein 10.0
NaCl 1.0
CaCO3 1.5
LOT 1399
Ration:
Wheat 72.5
Potatoes 25.0
NaCl 1.0
CaCO3 1.5
GRAMS
280
240
200
160
120
80
40
Lot 1453
2nd gen.
3rd gen.
Lot 1399
< 4 >
Weeks
Y = birth of young
Y
♀
♂
♀ N
♂ N

sources is capable of inducing good growth and moderately high fertility is shown by a comparison with Lot 1408, Chart 5. The limiting factor in Lot 1399 is fat-soluble A. There were two females in this group. One had two litters (eleven young) but destroyed them soon after birth. The other remained sterile.

Lot 1453 had a diet similar to that of Lot 1399 except that it contained 10 per cent of casein to supplement its protein content. This exerted marked improvement in their growth, fertility, and longevity. It is of special importance to compare these curves with those of Lots 1399 and 1408. The diet of Lot 1399 is very much improved either by the addition of protein or fat-soluble A. This illustrates a principle which many investigators have failed to grasp; *viz.*, that all the other factors in an experimental diet must accurately be evaluated before judgment can safely be formed concerning the quality of a food or preparation in respect to a factor under investigation. For example, the diet of Lot 1453 appears to contain sufficient fat-soluble A. Actually it is distinctly below the optimum in this substance. It contains the same amount of fat-soluble A as does the diet of Lot 1399, but this deficiency is not apparent in Lot 1453 for some months because of the excellent quality in all other factors. Deficiency in fat-soluble A, however, became manifest in the lowered nutritive value of the milk and caused injury to the young, especially in the third generation.

Two females in this group had collectively fifty-two young (seven litters) and weaned forty-two of them. One second generation female had twenty young (three litters) and weaned eleven. One third generation female had eleven young (two litters) and weaned nine of them. The young were strong and vigorous in most cases. A few of the second litter obtained from a third generation female were, however, quite undersized and puny.

Chart 5.—The curve of Lot 1462, Period 1, illustrates the behavior of young rats fed a diet in which all the inorganic salts were furnished by a mixture of wheat, casein, and potatoes. No growth could take place on this diet even though all factors other than the inorganic elements were properly constituted. In Period 2 when calcium carbonate and sodium chloride were added growth took place at once and at the maximum rate possible.

There were three females in this group. They had together forty-five young (six litters) and successfully weaned them all.

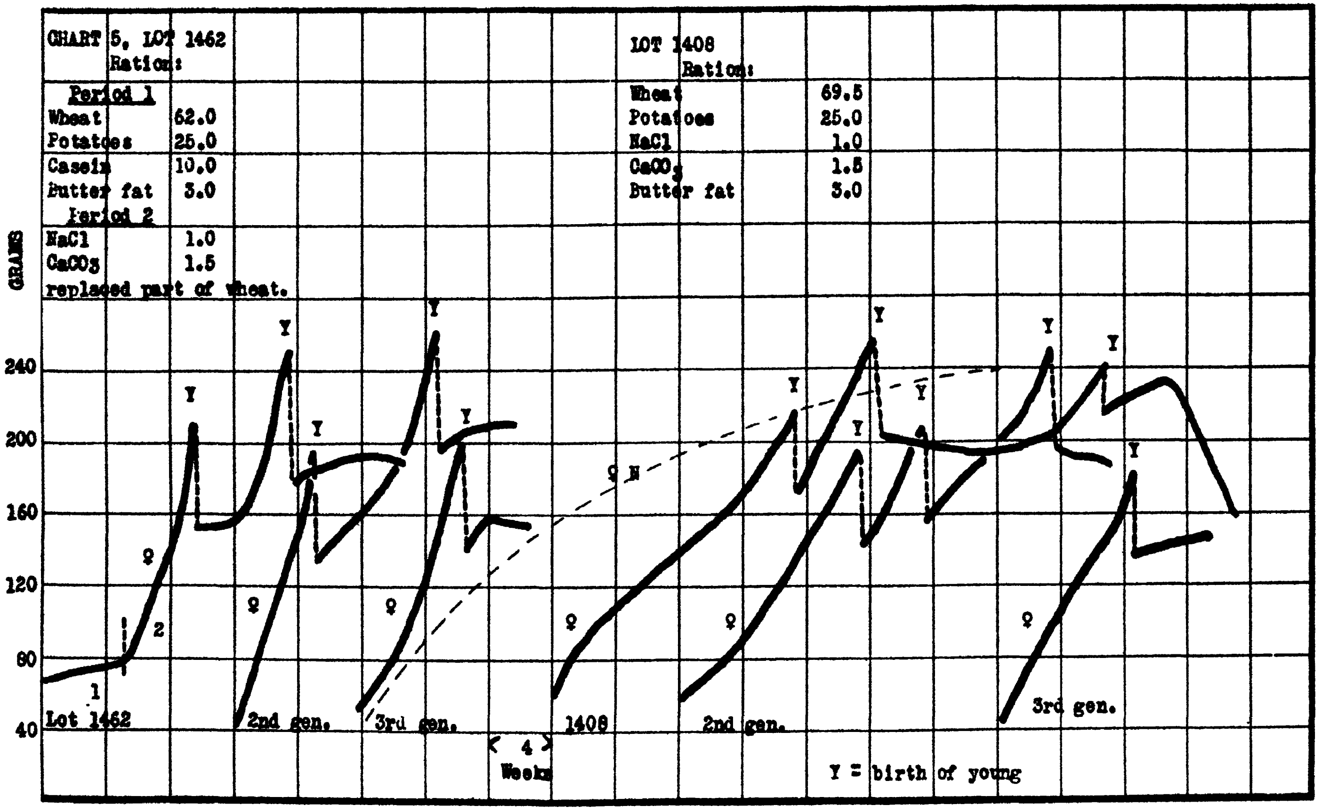
CHART 5, LOT 1462
Ration:
Period 1
Wheat 62.0
Potatoes 25.0
Casein 10.0
Butter fat 3.0
Period 2
NaCl 1.0
CaCO3 1.5
replaced part of wheat.
LOT 1408
Ration:
Wheat 69.5
Potatoes 25.0
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
GRAMS
240
200
160
120
80
40
Y
♀
1
2
N
Lot 1462
2nd gen.
3rd gen.
1408
2nd gen.
3rd gen.
< 4 >
Weeks
Y = birth of young

One second generation female was restricted to the family diet. She had two litters (sixteen young) and weaned them all. One third generation female had a litter of nine and reared them all. The young were always in excellent condition. This is an example of the success which we regularly see in successful growth and rearing of young when the mother's diet is satisfactory.

Lot 1408 derived all its protein from wheat and potato. The protein content of the diet was about 9 per cent, two-thirds of which was derived from wheat and one-third from potato. This amount of protein from these sources did not support growth at the maximum rate possible but the results indicate that there is some improvement in quality by combining proteins from these sources.

Two females in this group had twenty-one young collectively (four litters) and weaned eleven. One female in the second generation had eighteen young (three litters) and weaned eight of them. Two third generation females had fifteen young (two litters) of which twelve were weaned. The young in all these litters were small for their ages, and the nursing period was considerably prolonged in all cases in order to bring the young to a state of independence. Since these litters were not large in the first or second generation, it would seem probable that the failure of the young to thrive in a normal manner was due to faults in the quality rather than to lack of sufficient milk.

A comparison of the reproduction records of these groups (Lots 1462 and 1408) shows clearly that the infant mortality in the latter was the result of an inadequacy of the protein of the diet. On low protein diets, or diets containing protein of poor quality, we have regularly observed the necessity of a prolonged nursing period in order to fit the young to subsist on rations suitable for the adult.

Chart 6.—Lot 2150 illustrates a typical record of a group of rats fed from weaning time on a diet in which the protein was derived from peas and potatoes. Two-thirds of the protein was supplied by peas and the remainder by potato. The diet contained 9 per cent of protein (N × 6.25). Growth on this diet was approximately normal, showing that the mixed proteins of peas and potato at this plane of intake are far superior to pea proteins alone fed at this level (compare Lot 2376, Chart 1). Since dried potato is so

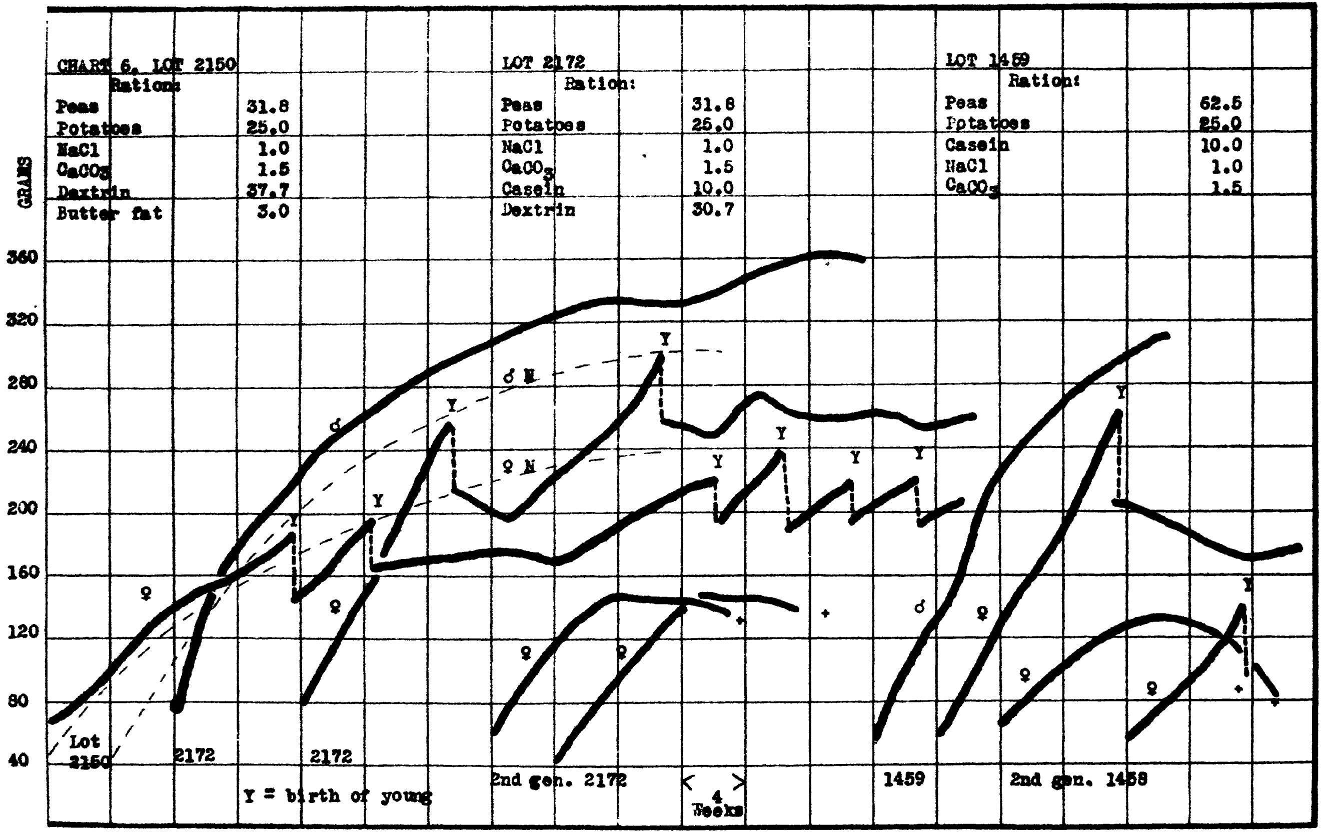

CHART 6. LOT 2150

Ration:

Peas	31.8
Potatoes	25.0
NaCl	1.0
$CaCO_3$	1.5
Dextrin	37.7
Butter fat	3.0

LOT 2172

Ration:

Peas	31.8
Potatoes	25.0
NaCl	1.0
$CaCO_3$	1.5
Casein	10.0
Dextrin	30.7

LOT 1459

Ration:

Peas	62.5
Potatoes	25.0
Casein	10.0
NaCl	1.0
$CaCO_3$	1.5

Y = birth of young

low in protein it is not possible to make a direct comparison of these mixed proteins with potato protein alone. That this content of protein from these sources is below the optimum is shown by a comparison of Lots 2150 and 2172.

There were three females in this group. They had collectively six litters (eighteen young) but none was weaned. Two other litters were born but were destroyed by the mother before the number was ascertained. The rats in this group looked very old at about the age of 1 year.

Lot 2172 had a diet similar to that of Lot 2150 except that it contained casein but no butter fat. The growth records were remarkably good, notwithstanding the fact that all the fat-soluble A was derived from peas (31.8 per cent) and potato (25 per cent). One male reached a weight of 360 gm., and a female, a non-pregnant weight of 245 gm.

There were two females in the group. They had together twenty-three young (four litters) and weaned thirteen of them. The young were inferior in appearance, but did not develop xerophthalmia. One mother, however, developed inflamed eyes. Four second generation females were restricted to the food mixture but all died after being subjected to this diet for 4 to 5 months without having any young.

Lot 1459 was fed a diet similar to that of Lot 2172, except that it contained about double the amount of peas. The first generation fed this diet grew well, but the fertility was low and they aged very early. They were ready to die at about a year.

There were two females in this group. They had collectively nineteen young (three litters) and weaned nine of them. Two females in the second generation were restricted to the diet. One became pregnant, but died while giving birth to young. The other died at the age of 6 months. This ration contained a high protein content (about 27.5 per cent), more than half of which was from peas. Pea protein when fed at high levels apparently causes damage to animals.[3] This diet (Lot 1459) contained considerably more fat-soluble A and protein in its content of peas than did that of Lot 2172. All other factors were equally satisfactory or to the advantage (water-soluble B) in the case of Lot 1459, yet the nutrition of the latter group was distinctly inferior to Lot 2172. These results harmonize with the observation of Osborne and Mendel[4] that young rats fed purified pea protein as the sole source of this factor steadily declined.

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[3] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 287.

The records of Lot 1459 should be compared with those of Lot 1468 (Chart 7). The diets of these groups were similar but the latter contained more fat-soluble A, and this resulted in marked improvement in their condition.

Chart 7.—The rats of Lots 1414 and 1405 derived their protein entirely from peas and potatoes. The pea protein constituted about 88 per cent of the total. The protein content of these food mixtures was 17 to 18 per cent. Even at this high level of intake these proteins are inferior to a mixture derived from wheat and potato, as is shown by a comparison of these groups with Lot 1408 (Chart 5).

Lot 1414 contained two females. They had collectively three litters (nineteen young) and weaned all of them. One female of the second generation had one litter of three and weaned them. Some of the original group were kept on the diet more than 10 months but by 8 months they appeared old looking.

Lot 1405 was fed a diet closely similar to that of Lot 1414 except that it contained no butter fat. The inferiority of these animals as compared with Lot 1414 was due entirely to the lower intake of fat-soluble A.

A striking illustration of the danger of misinterpreting data obtained in nutrition experiments of this character is afforded by a comparison of four sets of records in these charts. Lot 2172 (Chart 6) derived all its fat soluble-A from 31.8 per cent of peas and 25 per cent of potato. The protein was enhanced by the addition of casein and the necessary inorganic salts were added to complete the mineral content. During the first year of life these animals appeared normal except for the high infant mortality. Lot 1459 (Chart 6) had more peas, the same amount of potatoes and casein, and the same salts. It, therefore, secured more protein and more fat-soluble A than Lot 2172, yet the development of Lot 2172 was much more satisfactory. If we did not have the records of Lot 2172 we should have made the statement from the results of Lot 1405 that peas are very low in fat-soluble A. How-

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[4] Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307.

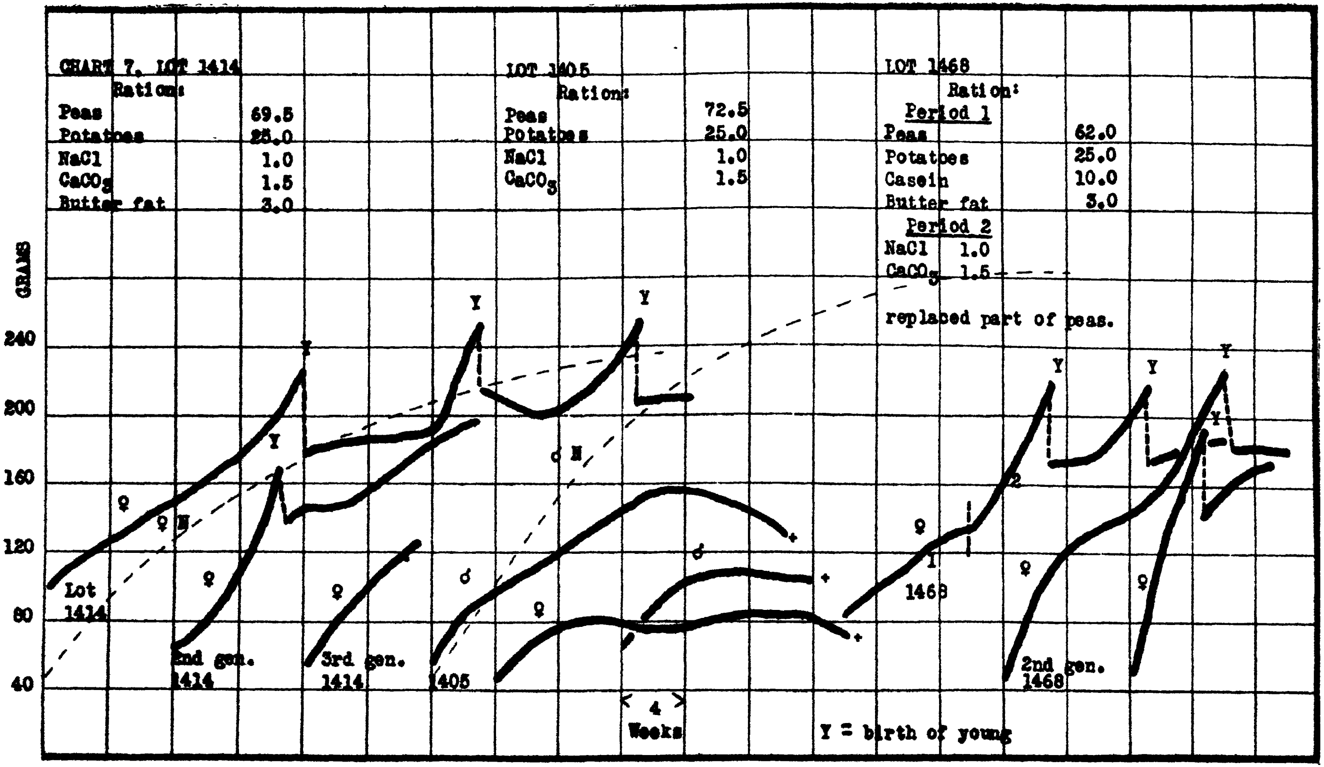
CHART 7. LOT 1414

ever, when the protein content was improved with casein, as in Lot 2172, the fat-soluble A derived from 31.8 per cent of peas and 25 per cent of potato had more beneficial effect than more than twice this amount taken with the same food mixture containing protein of lower biological value. The addition of butter fat (fat-soluble A) to Lot 1405, however, also made them develop distinctly better, as is shown in Lot 1414.

Lot 1405 (Chart 7) was fed 72.5 per cent of peas and therefore more fat-soluble A than either Lot 2172 or Lot 1459 (Chart 6), yet because the casein was omitted in Lot 1405 the animals were very inferior. The protein and phosphorus content of Lot 2172 is lower than that of Lot 1405, yet the growth was much better in the former than in the latter. This was due to superiority in the quality of the protein in the diet of Lot 2172, and this factor was of sufficient biological significance to make a decided difference in the well being of the two groups. These illustrate border-line cases of malnutrition. They represent a type of diet in which a small variation of quality upward in one or another factor, as protein or salts, may so modify the early history of the experimental animals as to make a particular factor, *e.g.* fat-soluble A content, appear in one set of results to be adequate for the physiological needs of the animals, while a variation in the quality downward either in protein or salts, such variation remaining at the same time well within physiological limits, may entirely change the deductions with respect to the value of the constant factor fat-soluble A.

This same idea is also brought out in Chart 8, Lot 1454, and also in Chart 11, Lot 1451 (see discussion under these charts).

Lot 1468 in Period 1 was fed a diet of peas, potatoes, casein, and butter fat. The growth of the animals on this food was in no case more than half normal (Period 1). In Period 2 sodium chloride and calcium carbonate were added. This led to a prompt response with growth and reproduction. It appears probable from these results that there is less injurious effect of high pea protein ingestion when its quality is enhanced by a casein addition than when the same pea protein content is taken without such improvement.

This group contained two females. They had collectively fourteen young (two litters) and weaned them all. Two second generation females had collectively twelve young (a litter each) and weaned eleven. These mothers were discarded after weaning their young. These animals presented a dirty appearance and were badly stained with their own urine.

Chart 8.—Lot 2149 was fed a diet, the protein of which was derived entirely from rolled oats and potato. It contained 9 per cent of total protein (N × 6.25), two-thirds of which was furnished by oats and one-third by potato. Other factors than protein were satisfactorily supplemented. Growth on this diet was somewhat below the optimum, but the protein derived from this combination is of better quality than a similar amount of oat protein alone. This combination does not possess so high a biological value as does a protein mixture from wheat and potato in the same proportions. This is illustrated by Lot 1408 (Chart 5).

Three females grew up on this diet. They had collectively thirty-one young (four litters) but none was weaned. The males were apparently in good condition at 7 months of age, but the females presented a poorer appearance. Those young which were not killed by the mother grew so little while nursing that it is evident that the nutritive value of the milk was low.

Lot 2173 derived all its fat-soluble A from rolled oats and potato. In other respects this diet was comparable with Lot 2149. The protein in the diet of Lot 2173 was enhanced by the addition of casein.

Three females grew up on this diet. One female had two litters, another, one litter, collectively eighteen young, and weaned but four of them. A third female became pregnant but died while giving birth to her young. The young were in a very poor condition. Two daughters of these mothers were restricted to the diet but never had any young. All these animals aged early. Their span of life was only 5 to 7 months.

Lot 1454 presents remarkable growth curves on a diet which all previous experience indicated to be very poor in fat-soluble A. All of this factor was derived from 62.5 per cent of rolled oats and 25 per cent of cooked dried potato. One male reached a weight of over 400 gm. Two females each had three litters, collectively forty-seven young, and weaned twenty-six. One daughter grew up on the diet and had two litters but lost them all during the first few days. These young were in poor condition and developed xerophthalmia in some cases. At the age of 11 months the animals appeared very old, but autopsy revealed nothing pathological.

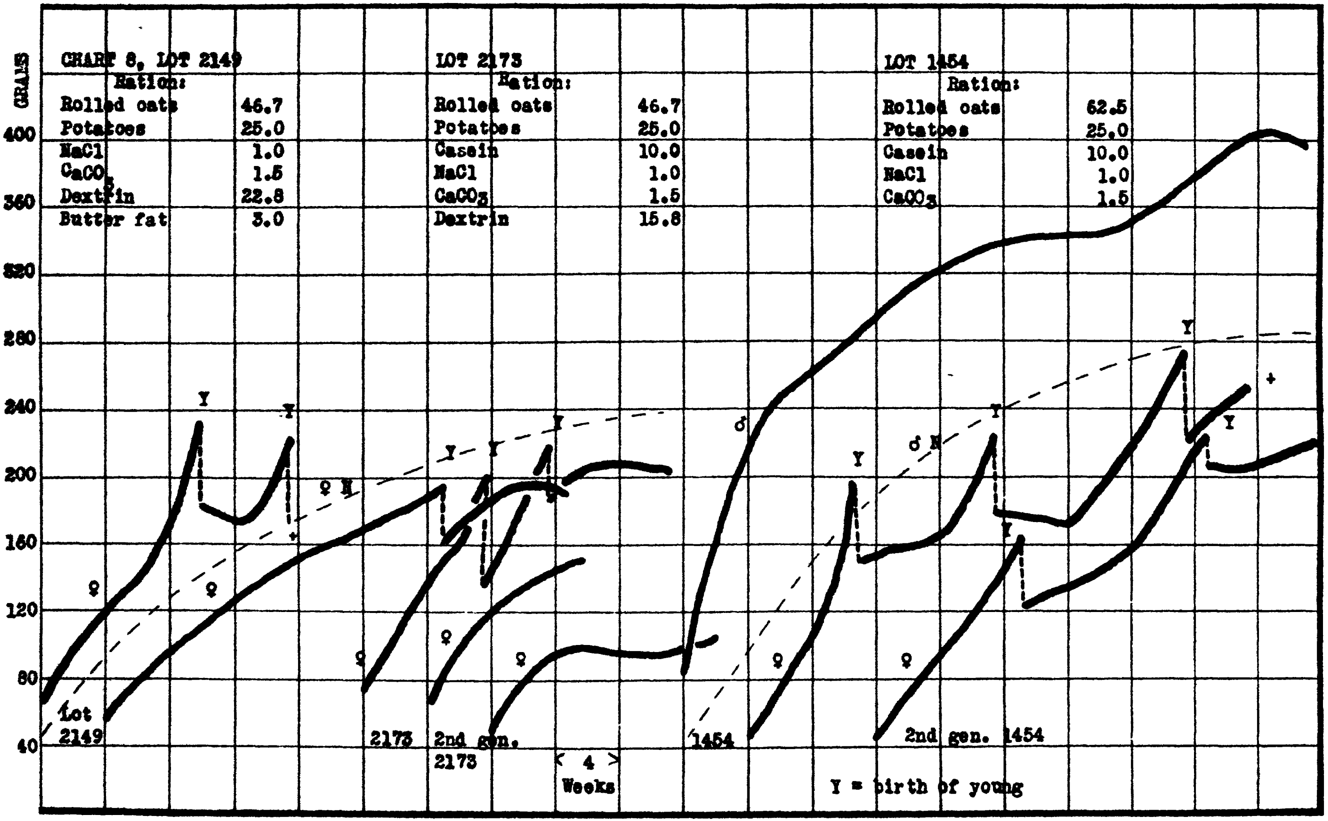
CHART 8, LOT 2149
Ration:
Rolled oats 46.7
Potatoes 25.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 22.8
Butter fat 3.0
LOT 2173
Ration:
Rolled oats 46.7
Potatoes 25.0
Casein 10.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 15.8
LOT 1454
Ration:
Rolled oats 62.5
Potatoes 25.0
Casein 10.0
NaCl 1.0
$CaCO_3$ 1.5
GRAMS
400
360
320
280
240
200
160
120
80
40
Y
Y
♀
♀
♀ N
+
Y
Y
Y
♀
♀
♀
♂
Y
♂ N
Y
Y
Y
Y
+
♀
♀
Lot 2149
2173
2nd gen. 2173
< 4 > Weeks
1454
2nd gen. 1454
Y = birth of young

Our experience in studying the dietary properties of the oat kernel has demonstrated it to be the poorest of the cereal grains in fat-soluble A. Steenbock and Gross report the white potato to be an inadequate source of this factor.[5] In interpreting the value of this diet with respect to fat-soluble A, Lot 1454 (Chart 8) and Lots 1409 and 1400 (Chart 9) should be compared. On the diet of Lot 1400 but little growth could take place, and xerophthalmia developed. Lot 1409, whose diet differed from that of Lot 1400 only in containing butter fat, was able to grow and reproduce. This was due specifically to the increased content of fat-soluble A. Lot 1454, whose diet differed from that of Lot 1400 only in that its protein content was improved by the addition of casein was also able to grow normally and reproduce and rear some young. The infant mortality was higher than that of Lot 1409. The content of fat-soluble A was the same in the diets of Lots 1400 and 1454. Such experimental results as these show the fallacy of deductions such as have been made by Hess and Unger[6] who fed infants during several months on a diet somewhat low in fat-soluble A but of excellent quality in other respects, and concluded that the factor fat-soluble A is not of great importance in practical human nutrition. Judgment on this matter must be based on a full appreciation of the difference between border-line malnutrition with its attendant grave dangers from infections or unfavorable reaction to any chance modification of the diet so as to reduce its quality in any way.

Chart 9.—Lot 1409 was fed a diet in which the protein was all derived from rolled oats and potato. The content of protein in the food mixture was about 12.5 per cent, about 16 per cent of this being derived from potato, and 84 per cent from oats. All other factors were adequately supplemented.

The rats which were confined to this diet never grew to the normal adult size. There were two females in this group. They had collectively eighteen young (four litters) but only two were weaned. The mothers destroyed the young. One of these rats died during the birth of her third litter. One second generation female grew up on the family diet and had fifteen young (two litters) and

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[5] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

[6] Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.

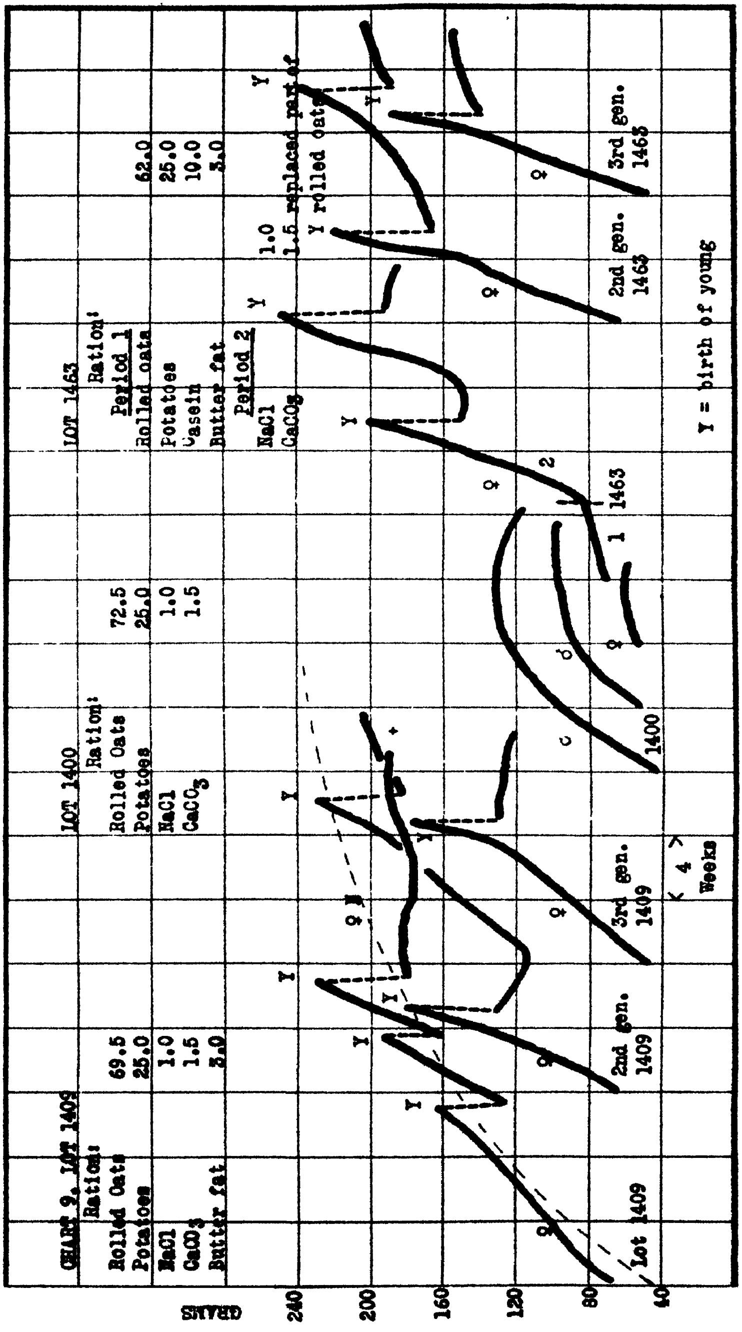
CHART 9. LOT 1409
Ration:
Rolled Oats 69.5
Potatoes 25.0
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
LOT 1400
Ration:
Rolled Oats 72.5
Potatoes 25.0
NaCl 1.0
CaCO3 1.5
LOT 1463
Ration:
Period 1
Rolled oats 62.0
Potatoes 25.0
Casein 10.0
Butter fat 3.0
Period 2
NaCl 1.0
CaCO3 1.5
replaced part of rolled oats
Y = birth of young
GRAMS
240
200
160
120
80
40
< 4 >
Weeks
Lot 1409
2nd gen. 1409
3rd gen. 1409
1400
1463
2nd gen. 1463
3rd gen. 1463
1
2
♀
♂
Y
+

weaned thirteen of them. One third generation female had one litter of six and successfully weaned them.

Lot 1400 was fed a diet like that of Lot 1409 except that it did not have a supplemental addition of fat-soluble A. Growth on this diet was slow and incomplete. The rats all died in 4 to 5 months after being placed upon the ration. Xerophthalmia developed in three of the four rats confined to this food mixture.

This diet with added fat-soluble A is capable of inducing nearly normal growth and supports reproduction and rearing of some young (Lot 1409). This ration with added protein has induced the maximum amount of growth in rats and supported reproduction and rearing of some young, although the infant mortality was high (Lot 1454, Chart 8). This illustrates the fact that one is not justified in drawing conclusions concerning the content of fat-soluble A in a foodstuff without furnishing evidence that all factors in the diet are accurately evaluated.

Lot 1463 was fed in Period 1 a diet consisting of rolled oats, potatoes, casein, and butter fat. The inorganic content of the diet was entirely derived from these sources. Practically no growth could take place on this food. In Period 2 sodium chloride and calcium carbonate were added. This led to marked response with growth to the full adult size.

Three females in this group had collectively thirty-eight young (six litters) and weaned thirty-six. One second generation female was restricted to the diet. She had fifteen young (two litters) of which fourteen were weaned. One third generation female had a litter of nine and weaned them all. These young were in all cases in excellent nutritive condition.

A comparison of the reproduction records and infant mortality of Lot 1463 with those of Lot 1454 (Chart 8) shows clearly the beneficial effects of a liberal supply of fat-soluble A. This is shown by a mortality among the young of 45 per cent in Lot 1454 in the first generation and of 100 per cent in the second generation.

In Lot 1463, 95 per cent of the young were weaned. The appearance, size, and condition of the young of these two groups presented as marked a contrast as did the infant mortality.

Chart 10.—Lot 1406 derived all its protein from 69.5 per cent of maize and 25.0 per cent of potatoes. All other factors were

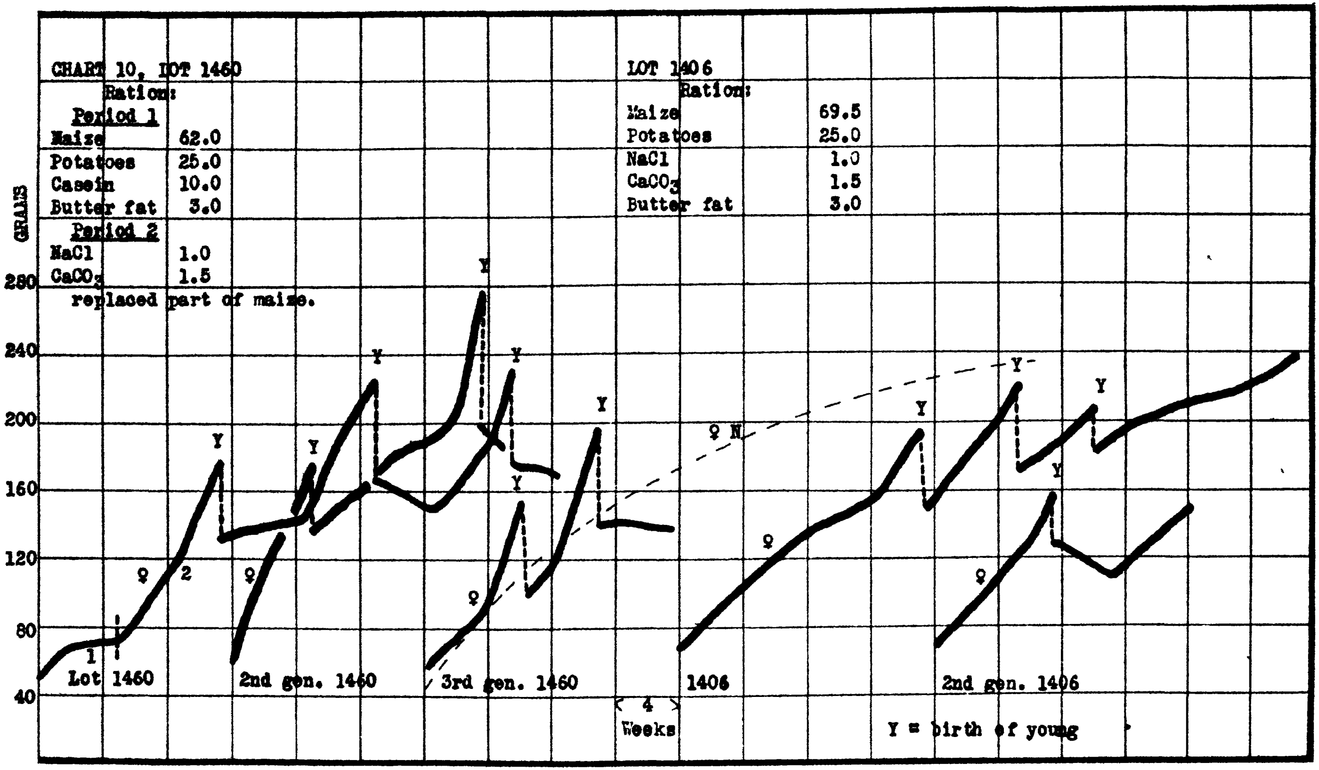
CHART 10, LOT 1460
Ration:
Period 1
Maize 62.0
Potatoes 25.0
Casein 10.0
Butter fat 3.0
Period 2
NaCl 1.0
CaCO$_3$ 1.5
replaced part of maize.
LOT 1406
Ration:
Maize 69.5
Potatoes 25.0
NaCl 1.0
CaCO$_3$ 1.5
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
Y
♀
1
2
♀ N
Lot 1460
2nd gen. 1460
3rd gen. 1460
1406
2nd gen. 1406
4 Weeks
Y = birth of young

satisfactorily adjusted by suitable additions. Growth was somewhat retarded because of the quality in the protein factor.

Three females grew on this diet. They had collectively thirty-five young (six litters) and weaned eleven of them. Two second generation females were restricted to this diet. One had a litter of two young and after a long nursing period weaned them in poor condition. The other remained sterile. The animals in this group were always somewhat undersized and of poor appearance.

Lot 1460 in Period 2 had a diet like that of Lot 1406 except that it contained 10 per cent of casein. In Period 1 no inorganic salts were added. Without these growth was suspended, but with them the animals grew rapidly. This shows that in Lot 1406 the quality and amount of protein was the limiting factor.

Three females grew up on this diet. They had collectively fifty young (seven litters) and weaned forty-eight of them in good condition. One second generation female had seventeen young (two litters) and weaned fifteen of them. One third generation female had fifteen young (two litters) and weaned seven. The young were vigorous and active but their coats were not as glossy and well kept as those of other rats which we have observed. The contrast between these young and those of Lot 1406, on a lower protein plane, was very marked.

Chart 11.—Lot 1397 derived both protein and fat-soluble A entirely from maize and potato. The maize used in these experiments was of the yellow variety. A comparison of these growth curves with those of Lot 1451 (Chart 11) shows that notwithstanding the high content of yellow maize in this food mixture, protein was the first limiting factor in preventing normal development. Lack of sufficient fat-soluble A was, however, a very important deficiency in this diet, as shown by comparing the curves of Lot 1397 with those of Lot 1406 (Chart 10).

There was more fat-soluble A in the diet of Lot 1397 than in that of Lot 1451, yet the satisfactory content of protein in the latter diet made it appear, in the absence of a complete series of biological tests, to contain a satisfactory amount of this factor. Although certain yellow maizes doubtless contain more fat-soluble A than certain white varieties it seems to us that there has been some exaggeration of the value of yellow maize as a source of this

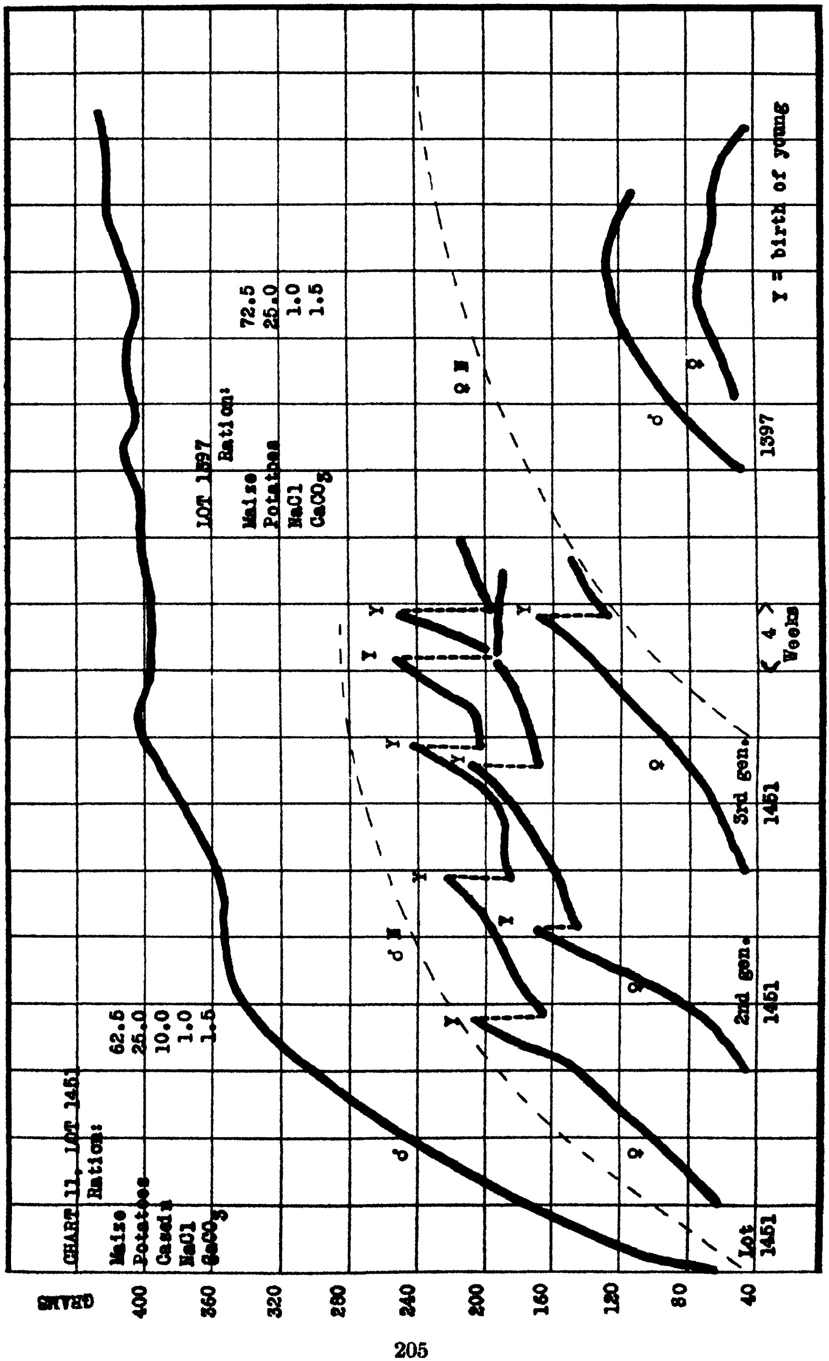
CHART 11. LOT 1451
Ration:
Maize 62.5
Potatoes 25.0
Casein 10.0
NaCl 1.0
CaCO3 1.5
LOT 1597
Ration:
Maize 72.5
Potatoes 25.0
NaCl 1.0
CaCO3 1.5
GRAMS
400
360
320
280
240
200
160
120
80
40
♂
♀
♂ N
♀ N
Y
Lot 1451
2nd gen. 1451
3rd gen. 1451
1597
4 Weeks
Y = birth of young

factor.[7] If we were limited to experiments with diets of the type fed Lots 1451 (Chart 11) and 1454 (Chart 8) we could easily have fallen into the error of interpreting the results as indicating that diets containing 60 to 70 per cent of yellow maize or even rolled oats supplemented with potato to the extent of 25 per cent of the food mixture would prove satisfactory in their content of fat-soluble A. More carefully planned experiments eliminate the danger of error in studies of this nature.

Lot 1451 contained two females. They had collectively sixty-four young (eight litters) and weaned fifty-nine. Two second generation females had five litters (twenty-seven young) and weaned twenty-two. Two third generation females had collectively eleven young (two litters) and weaned ten. All the young were in good condition, but in a few cases the hair was short and silky in appearance.

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[7] Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81.

SUPPLEMENTARY PROTEIN VALUES IN FOODS.

IV. THE SUPPLEMENTARY RELATIONS OF CEREAL GRAIN WITH CEREAL GRAIN; LEGUME SEED WITH LEGUME SEED; AND CEREAL GRAIN WITH LEGUME SEED; WITH RESPECT TO IMPROVEMENT IN THE QUALITY OF THEIR PROTEINS.

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Those who have discussed the world's food supply during the past few years have talked in great measure about the cereal grains as the most important and economical source of human food. Indeed, for many years the cereals and legume seeds have been increasingly cultivated both in Europe and America to meet the needs of their rapidly increasing populations. Bread grains have actually become the staff of life of a large part of the human family. It is now recognized by students of nutrition that the cereal grains individually and collectively are incomplete foods.[1] The war has taught through tragic experience that children cannot be kept alive for a very long period on such a food supply, although the requisite calories and energy may be supplied. The history of scurvy furnishes numerous examples of the dangers attending the restriction of the diet of adults to cereal products, legume seeds, and meats. Nevertheless, statisticians and persons in charge of feeding large groups of people in armies, prisons, asylums, hospitals, and labor camps, still in many instances show lack of appreciation of the dangers of limiting people to certain types of restricted diets.

It was natural that in the early efforts to discover the fundamental truths regarding the nutritive requirements of mammals, investigators should have attached much importance to the rate of growth during limited periods by young animals fed diets of different types. It was spectacular to observe complete failure in one case on a diet with which a chemist could find nothing amiss, and successful growth for weeks or months on another diet which according to older views should have been no more satisfactory than the first. With advancing knowledge, however, it became possible to interpret the cause of failure or success with any given diet, and new goals were set up by those who were able to make progress in nutrition studies. In our laboratory we have for several years sought to approach the solution of the problem of what constitutes the optimum diet for the purpose of promoting growth, of supporting highest fertility, greatest success in rearing young, and of preserving for as long a period as possible the characteristics of youth, and of extending to the extreme limit the span of life. With this objective we have come, as the result of much experience, to question whether the extension of the use of cereal grains in the diet of man has not already passed the limit of safety. At least it is more necessary now than formerly that the remaining components of the diet should be chosen with knowledge and care in order to correct the deficiencies of the cereal, tuber, and muscle meat mixture, or the bread, potato, and meat type of diet which has become so prominent a feature of the nutrition of many American and European families at the present time. It has been already pointed out that the cereal grains, legume seeds, and muscle tissue meats are too poor in calcium, sodium, chlorine, and fat-soluble A to meet the physiological requirements of a mammal. Mixtures of these in any variety are little, if any, better sources of the essential mineral elements or of fat-soluble A than are the individual foods themselves.[2]

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[1] McCollum, E. V., The newer knowledge of nutrition, New York, 1918. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.

Primitive man ate everything he could secure which was edible. His animal food included the flesh of such game as he could catch, and also fish, eggs, birds, shell-fish, insects, etc. Among the vegetable products which he doubtless ate were fruits, berries, fleshy roots, nuts, and a few other seeds of plants, among which were the seeds of those grasses which have since been developed into our cereal crops. There are relatively few regions where nuts are sufficiently abundant to furnish a regular article of diet for a sparse population during even a few months in the year, and the supply of cereal grains was even more inadequate. The cereal grains are the seeds of several grasses. In a country where no agriculture was practised, grasses would be cropped by grazing animals and the development of seed greatly interfered with. As Huntington points out, extensive agriculture was impossible until after animals were domesticated.[3] Such seed as was produced was born on isolated and scattered stems, and would be difficult to harvest in appreciable quantities. One exception was the wild rice plant which grew in the water and was therefore protected to some extent from grazing animals. It was more abundant in certain places than the seeds of any land grasses were likely to be in the unmolested fruiting condition. Rice was therefore harvested from very early times in Asia and in some of the northern states of America. Even in these favored regions of shallow lakes and rivers, however, rice never formed a principal constituent of the diet of the Indians, but only an adjuvant in the fall and early winter. Grass seeds of the type of the cereal grains are always eagerly sought for at ripening by birds, and the harvest time would naturally be short. Maize was never a prominent article of diet among the Indians, but only served to vary their otherwise carnivorous food supply. All the higher apes eat more or less of tender leaves which have mild flavors. After man reached a stage of development where food was regularly cooked he was able to eat coarse vegetables of the leafy type in greater variety and in larger amounts than when he had to eat them raw, because of the difficulty of digesting some of them. Pot-herbs early became a regular part of the diet of man as he advanced toward civilization. They are today the outstanding feature of the diet of the Chinese and Japanese.

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[2] McCollum, E. V., Proceedings of the Institute of Medicine of Chicago, 1920, iii, 13.

The great increase in the consumption of cereal grains in various forms as flours, corn-meal, corn grits, rolled oats, and in the form of the many breakfast foods found on the market as human food is an incident in connection with the development of modern industry, and the change from a rural to an urban life. This has forced a great part of the population to depend upon the remainder for food while they operate the machinery of industry. The land yields calories and protein in greatest abundance when farmed to cereals and leguminous plants, rather than when used for the production of milk or meat. This type of farming has therefore been encouraged, and wheat and maize production has been stimulated year by year to higher and higher levels until the world's capacity has nearly been reached in this respect. This great consumption of grains has changed the character of the diet profoundly from what it has ever been before in human history, and in a manner which tends to undermine the vitality. The national dietetic sin of America and many parts of Europe has grown to be close adherence to a meat, bread, and potato diet, or other foods which have similar dietary properties. When it is remembered that the cereal grains are now all but universally decorticated and degerminated for the purpose of producing products which can be kept without commercial hazard, and that these are decidedly poorer in their dietary properties than are the seeds from which they were milled, the situation can easily be appreciated. The diet of cereals, muscle tissue meats, and tubers (the meat, bread, and potato type) is not satisfactory for the nutrition of man or animals, and is of distinctly poorer quality when highly milled products are used in abundance.[4]

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[3] Huntington, E., Civilization and climate, New Haven, 1916.

We wish to emphasize in connection with this study of the manner in which the cereal grains supplement, or rather fail to supplement, the proteins of other cereal grains and legume seeds, the *fact that the widespread use of this class of foods represents an innovation in man's diet, and one which is not for the best.* The cereals may well be used as articles of human food, but it is wiser to utilize more of the land for the extension of the dairy industry in order to increase the supply of milk and other dairy products, than to seek to extend as far as possible the production of crops which yield the greatest returns in such food units as the chemist has long recognized, but which fall short of the requirements of mammals in respects which we have but recently been able to appreciate.

When one studies the charts described in this paper it is difficult to avoid the conclusion that a diet composed too largely of cereal products, and not satisfactorily supplemented by foods which correct their deficiencies, will tend to lower the vitality and promote the early development of senile characters. We cannot agree with Daniels and Nichols that the consumption of legume seeds such as the soy bean should be increased.[5] It is much better to use these seeds for feeding dairy cows, for their deficiencies can be made good by the latter through the consumption of forage plants, and used as a source of milk formation. Milk forms the most satisfactory corrective food to make good the deficiencies of the cereal, muscle meat, and tuber diet now in such widespread use.

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[4] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxviii, 113.

SUMMARY.

The charts presented in this paper bring out the following facts: Earlier observations on the inferior character of the proteins of the legume seeds have been confirmed. It is further shown that protein mixtures derived from two such seeds, the selection being made from navy or soy beans or peas, are little, if any, better than the proteins of the individual seeds, which compose the mixture when fed as the sole source of nitrogen.

Two cereal grains when combined fail to form a protein mixture which is markedly superior to the same amount of protein from a single grain for the nutrition of the rat.

In certain instances the improvement in the quality of the proteins is decidedly great when a cereal grain is supplemented with a legume seed. Conspicuous examples of such enhancement of proteins are wheat and navy beans (Chart 12), and wheat and peas (Chart 13).

Table 1 presents the records of the weights of several litters of young rats, the mothers of which were confined to diets which were essentially comparable in quality in all factors other than protein (Lots 2369, 2365, 2370, and 1236). The rate of growth of the nursing young served as an index to the extent to which the protein moiety of the food mixture met the needs of the mother for her own maintenance and for milk production.

Lot 2369 should be contrasted with Lot 2069. Each litter contained four young. Lot 2369 derived its protein (9 per cent) from maize and peas. Growth was retarded, indicating that the quality of the milk was below the optimum. Lot 2069 derived its

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[5] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

TABLE I.

This table shows typical records of nursing mothers on diets containing 9 per cent of protein from different sources, as contrasted with others on diets equally well constituted in respect to all other factors and containing higher protein contents of good quality.

Lot No.	Weight of ♀ when young were born	Weight of ♀ 10 to 18 days later		Weight of ♀ when through nursing.		No. of young.	Weight.						Source and content of protein.
	gm.	*days*	*gm.*	*days*	*gm.*		*days*	*gm.*	*days*	*gm.*	*days*	*gm.*	
2369	144	18	150	46	120	4	18	71	31	107	46	123	Maize 6 per cent protein, peas 3 per cent protein (see Chart 9).
2069	215	10	232	22	205	4	10	100	22	197			Degerminated cereals, peas beans, steak, and cabbage (18.2 per cent protein).
2365	173	18	157	45	146	5	18	70	30	102	45	210	Barley 6 per cent protein, navy beans 3 per cent protein (see Chart 8).
2370	192	12	170	37	170	5	12	74	33	160	37	175	Wheat 6 per cent protein, peas 3 per cent protein.
2153	175	15	182	28	192	5	15	137	28	277			Degerminated cereals, peas, beans, steak (19 per cent protein).
2370	177	20	145	44	175	7	12	81	33	167	44	250	Wheat 6 per cent protein, peas 3 per cent protein (see Chart 13).
1236	142			40	146	7	16	133	30	286	40	544	Steak 50 per cent, salts and butter fat, 35 per cent protein.

Lot 2069.	
Bolted flour	30.0
Corn-meal	15.5
Rice	8.0
Rolled oats	8.0
Peas	8.0
Navy beans	8.0
Round steak (cooked)	10.0
Cabbage (dry)	10.0
NaCl	1.0
$CaCO_3$	1.5

Lot 2153.	
Bolted flour	30.0
Corn-meal	19.5
Rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Round steak (cooked)	10.0
NaCl	1.0
$CaCO_3$	1.5

Lot 1236.	
Round steak (cooked)	50.0
NaCl	1.0
KCl	1.0
$CaCO_3$	1.5
Dextrin	43.5
Butter fat	3.0

protein from a variety of sources, and the amount was approximately double that in the diet of Lot 2369. These young were more than 25 per cent heavier at 10 days of age than those of Lot 2369 were at 18 days.

The nursing mother in Lot 2365 derived her protein (9 per cent) from barley and navy beans. The young nursing this mother fell far behind the growth of those which nursed a mother of Lot 2370, which derived its protein (9 per cent) from wheat and peas. The former litter of five young at 18 days weighed collectively 70 gm. The latter at 12 days weighed 74 gm. This difference in the nutrition of these nursing young was solely due to the difference in quality of protein in the two food mixtures. Other equally interesting comparisons can be seen in the table.

Lots 2069 and 2153 received 18 and 19 per cent of protein, respectively, and from a variety of sources. Their diets were, however, decidedly below the optimum in their content of fat-soluble A, and for this reason would not serve to maintain the vitality of a family through successive generations. A further discussion of this type of diet and its effect on quality of milk secreted and on the span of life will be given in a later paper.

Chart 1.—The curves presented in this chart are typical of groups of rats which were fed from an early age on diets which derived their protein contents from: (1) peas; (2) soy beans; (3) peas and navy beans; and (4) soy beans and navy beans. In each case the content of protein in the diets was 9 per cent. Lot 2367 derived two-thirds of its protein supply from peas and the remainder from navy beans. Lot 2366 derived two-thirds of its protein from soy beans and one-third from navy beans. Deficiencies in the

CHART 1.

LOT 2376

Ration: Period 1	
Peas	40.8
NaCl	1.0
$CaCO_3$	1.5
Dextrin	53.7
Butter fat	3.0

LOT 2510

Ration: Period 1	
Soy beans	24.9
Salts (185)	3.7
NaCl	1.0
$CaCO_3$	1.5
Dextrin	65.9
Butter fat	3.0

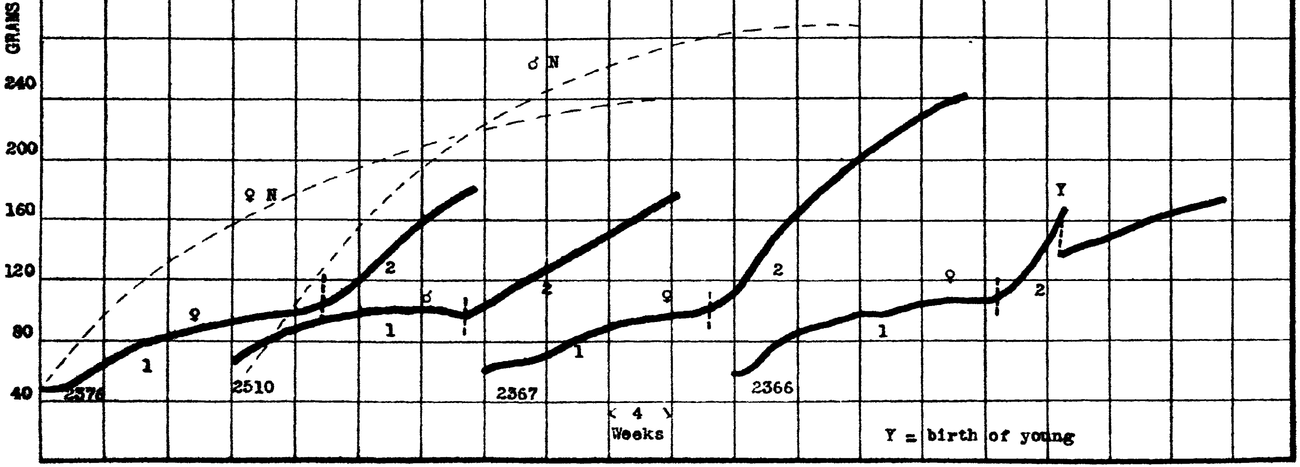
Lot 2367

Ration: Period 1	
Peas	27.2
Navy beans	13.6
NaCl	1.0
$CaCO_3$	1.5
Dextrin	53.7
Butter fat	3.0

LOT 2366

Ration: Period 1	
Soy beans	16.6
Navy beans	13.6
NaCl	1.0
$CaCO_3$	1.5
Dextrin	64.3
Butter fat	3.0

Period 2: Lots 2376, 2367, and 2366 had 10 per cent of casein added to the diet. Lot 2510 had 5 per cent of casein added.



Y = birth of young

mineral and fat-soluble A content of the seeds were corrected by suitable additions, and accordingly the character of the growth curves depended on the quality of the protein.

These records show that combinations of proteins of peas and navy beans or combinations of proteins of soy beans and navy beans, are not superior in their biological values to the proteins of any one of these legume seeds when fed as the sole source of protein in diets of closely comparable composition in all respects other than the source of protein.

Of the females in Lot 2366 only one ever had any young. This rat had a litter of four which were destroyed soon after birth.

Chart 2.—The curves shown in this chart represent typical growth records of several groups of rats fed on diets deriving their proteins in each case from two legume seeds. The combinations include navy beans and peas; soy beans and peas; and navy beans and soy beans. Lot 2368 contained navy bean proteins as the sole source of nitrogen in a diet otherwise comparable in all respects to the diets of the animals whose curves are shown in the chart.

In Period 2, 10 per cent of casein was added to the diets. In every case this led to a marked response with growth, but doubtless at a slower rate than would have been the case if the animals had not suffered a long period of stunting.

It is a most remarkable fact that the legume seeds when combined with each other do not form protein mixtures which are superior to the proteins of the individual seeds themselves. This is, apparently, to be explained on the assumption that a certain amino-acid which is present in such small amount as to be the limiting factor in determining the biological value of the proteins of these seeds, is the same in each of the legume seeds used in these experiments. Otherwise, it seems that a supplementary effect should have been observed in some of these combinations. It is by no means demonstrated that all the indispensable amino-acids have been identified, but if one may judge from the recorded data relating to yields of various amino-acids it seems suggestive that the low content of cystine yielded by all legume proteins may be the explanation for their failure to enhance each others values when combined.[6] We shall later discuss experimental data shedding light on this point.

[6] Sure, B., *J. Biol. Chem.*, 1920, xliii, 443.

CHART 2

LOT 2375 Ration: Period 1	
Navy beans	27.2
Peas	13.6
NaCl	1.0
$CaCO_3$	1.5
Dextrin	53.7
Butter fat	3.0

LOT 2374 Ration: Period 1	
Soy beans	16.6
Peas	13.6
NaCl	1.0
$CaCO_3$	1.5
Dextrin	64.3
Butter fat	3.0

LOT 2383 Ration: Period 1	
Navy beans	27.2
Soy beans	8.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	59.0
Butter fat	3.0

LOT 2382 Ration: Period 1	
Peas	27.2
Soy beans	8.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	59.0
Butter fat	3.0

LOT 2368 Ration: Period 1	
Navy beans	40.8
NaCl	1.0
$CaCO_3$	1.5
Dextrin	53.7
Butter fat	3.0

Period 2: 10 per cent of casein was added to ~~the diet in each~~ case.

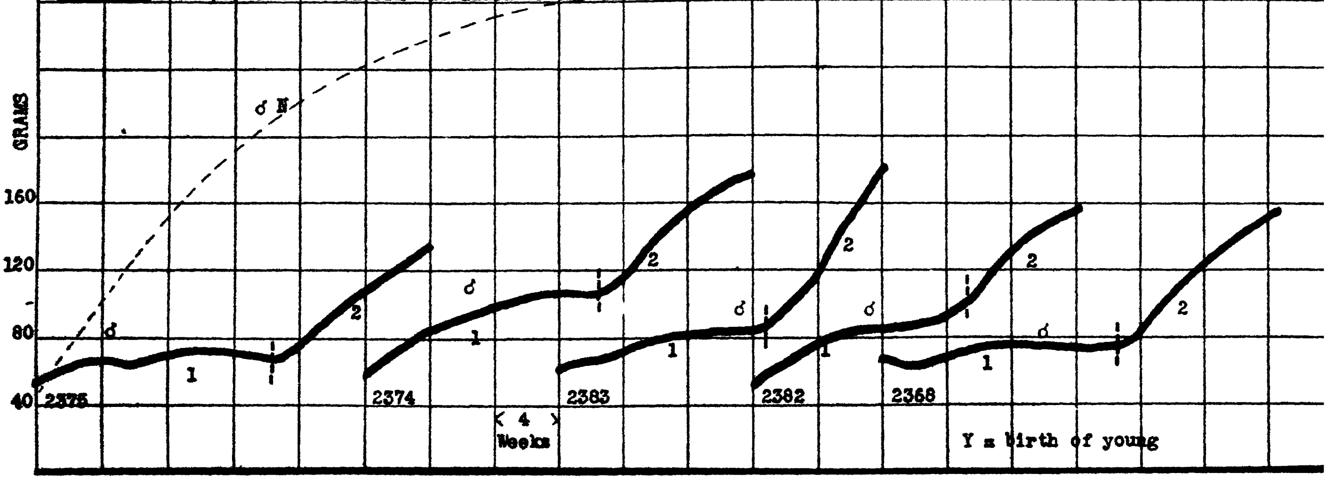


Chart 3.—The curves in this chart illustrate the growth and reproduction records of rats fed proteins derived from two cereal grains. All factors other than protein were made satisfactory by suitable additions. In all cases the protein content of the diet was 9 per cent.

Lot 2551 derived 6 per cent of protein from rolled oats and 3 per cent from maize. Growth was only fairly satisfactory. All remained undersized. The females reached non-pregnant weights of about 150 gm. There were two females in the group but neither had any young.

Lot 2550 derived 6 per cent of protein from rolled oats and 3 per cent from wheat. These animals were undersized and not in very good condition. Three females had eleven young (two litters) but all died during the nursing period. One litter of five weighed 52 gm. at 10 days of age and was, therefore, stunted and in poor condition. At 19 days they were all dead. The stunted condition and early death of these young were probably due to the poor quality of the milk of the mother.

Lot 2547 derived 6 per cent of protein from maize and 3 per cent from wheat. These rats were undersized and their fertility was low. The growth curves of this group were not so satisfactory as we have regularly seen in animals fed a comparable diet containing 9 per cent of protein derived entirely from wheat, but were slightly better than could be secured with 9 per cent of maize protein. There were two females in this group. One remained sterile. The other had a single litter of five young, but destroyed them within a week.

Lot 2546 secured its protein entirely from wheat (6 per cent) and maize (3 per cent). While the growth curves were but slightly better in general than those of Lot 2547, the animals were in somewhat better condition.

There were two females in this group. One was sterile, while the other had but ten young (two litters) and weaned two. The nursing period was prolonged to 73 days before the young were sufficiently developed to make it possible for them to lead an independent existence on the family diet. At the age of 73 days the litter of five had been reduced to two and these weighed together but 103 gm. and were very inferior.

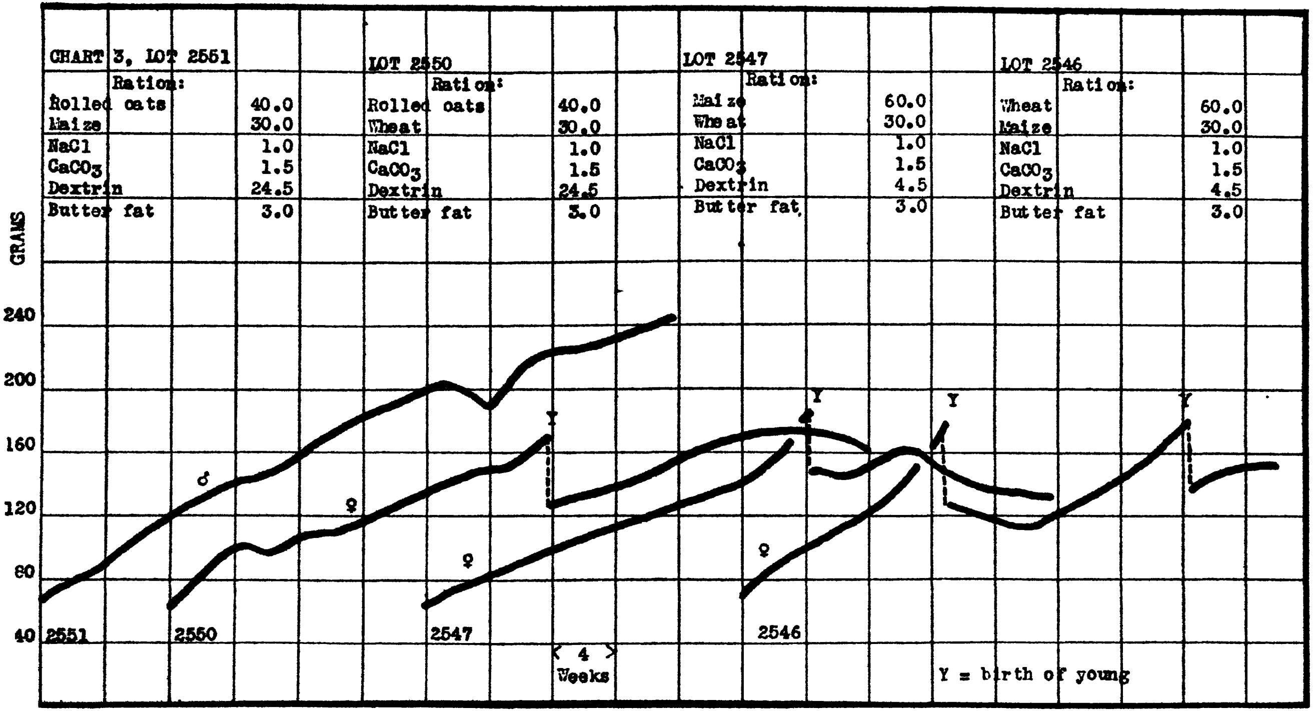


CHART 3, LOT 2551
Ration:
Rolled oats 40.0
Maize 30.0
NaCl 1.0
CaCO3 1.5
Dextrin 24.5
Butter fat 3.0
LOT 2550
Ration:
Rolled oats 40.0
Wheat 30.0
NaCl 1.0
CaCO3 1.5
Dextrin 24.5
Butter fat 3.0
LOT 2547
Ration:
Maize 60.0
Wheat 30.0
NaCl 1.0
CaCO3 1.5
Dextrin 4.5
Butter fat 3.0
LOT 2546
Ration:
Wheat 60.0
Maize 30.0
NaCl 1.0
CaCO3 1.5
Dextrin 4.5
Butter fat 3.0
GRAMS
240
200
160
120
80
40
♂
♀
♀
♀
Y
Y
Y
Y
2551
2550
2547
2546
< 4 >
Weeks
Y = birth of young

Chart 4.—Lot 2346 was fed a mixture of wheat and oat proteins in such proportions that each cereal contributed 4½ per cent of protein to the diet. These animals grew decidedly better than did those of Lot 2550 (Chart 3), which had the same protein intake but derived to the extent of two-thirds of the total from rolled oats. In oat and wheat mixtures the protein appears to be of better quality the higher the proportion of wheat.

There were two females in this group. They had together thirty-three young (seven litters) and weaned nine of them. Three second generation females remained sterile on this diet. The fertility records of this group were as much superior to those of Lot 2550 as were the growth curves. The nursing period here was again long. A litter of seven young was reduced to five by 13 days. The remaining five weighed collectively at this time 48 gm. At 32 days the five weighed 101 gm. and were in rather poor condition. At 48 days the five weighed 148 gm.

Lot 2344 was fed 9 per cent of protein derived from wheat and maize in equal proportions. The curves are better than those of Lot 2547 (Chart 3) which had 6 per cent of maize and 3 per cent of wheat proteins, respectively.

Two females had sixteen young (three litters) and weaned seven of them. These were very small for their ages. Three young at 32 days weighed 62 gm. and were poorly nourished. Two females in the second generation remained sterile.

Chart 5.—Lot 2345 derived its protein (9 per cent of the diet) from maize and rolled oats in equal proportions. This mixture was somewhat better than that of Lot 2551 (Chart 3) in which the oats and maize supplied 6 and 3 per cent of the protein, respectively.

Two females had thirty-five young (six litters) and weaned ten. Two other litters were born but their number were not ascertained because their mothers destroyed them as soon as they were born. One second generation female had fifteen young (three litters) and weaned three. The other remained sterile. The nursing period was very long. A litter of eight young was reduced by death to four by the 13th day. The remaining four weighed but 35 gm. At 62 days the three of these which remained weighed 92 gm. collectively. They were in an inferior condition.

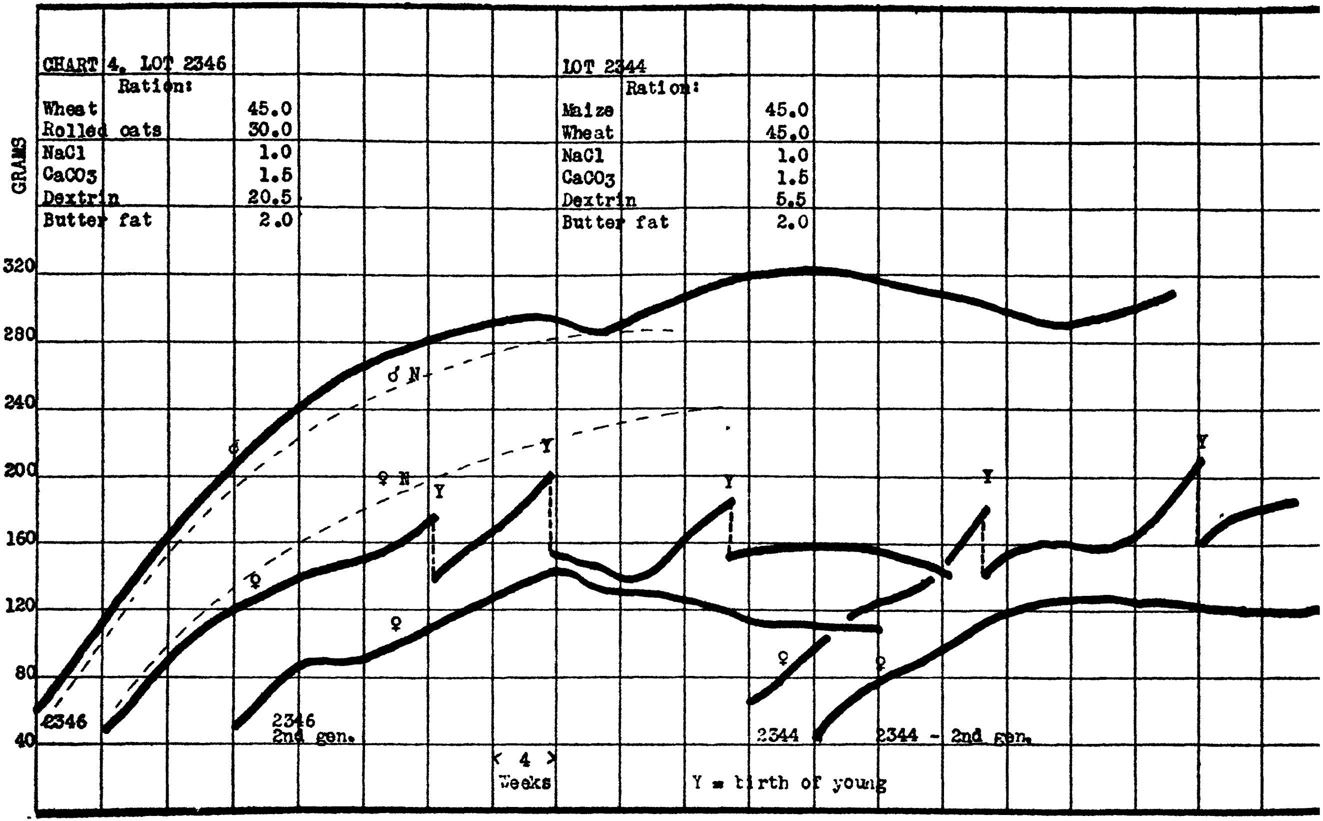


CHART 4. LOT 2346
Ration:
Wheat 45.0
Rolled oats 30.0
NaCl 1.0
CaCO3 1.5
Dextrin 20.5
Butter fat 2.0
LOT 2344
Ration:
Maize 45.0
Wheat 45.0
NaCl 1.0
CaCO3 1.5
Dextrin 5.5
Butter fat 2.0
GRAMS
320
280
240
200
160
120
80
40
♂ N
♀ N
♂
♀
Y
2346
2346
2nd gen.
2344
2344 - 2nd gen.
< 4 >
Weeks
Y = birth of young

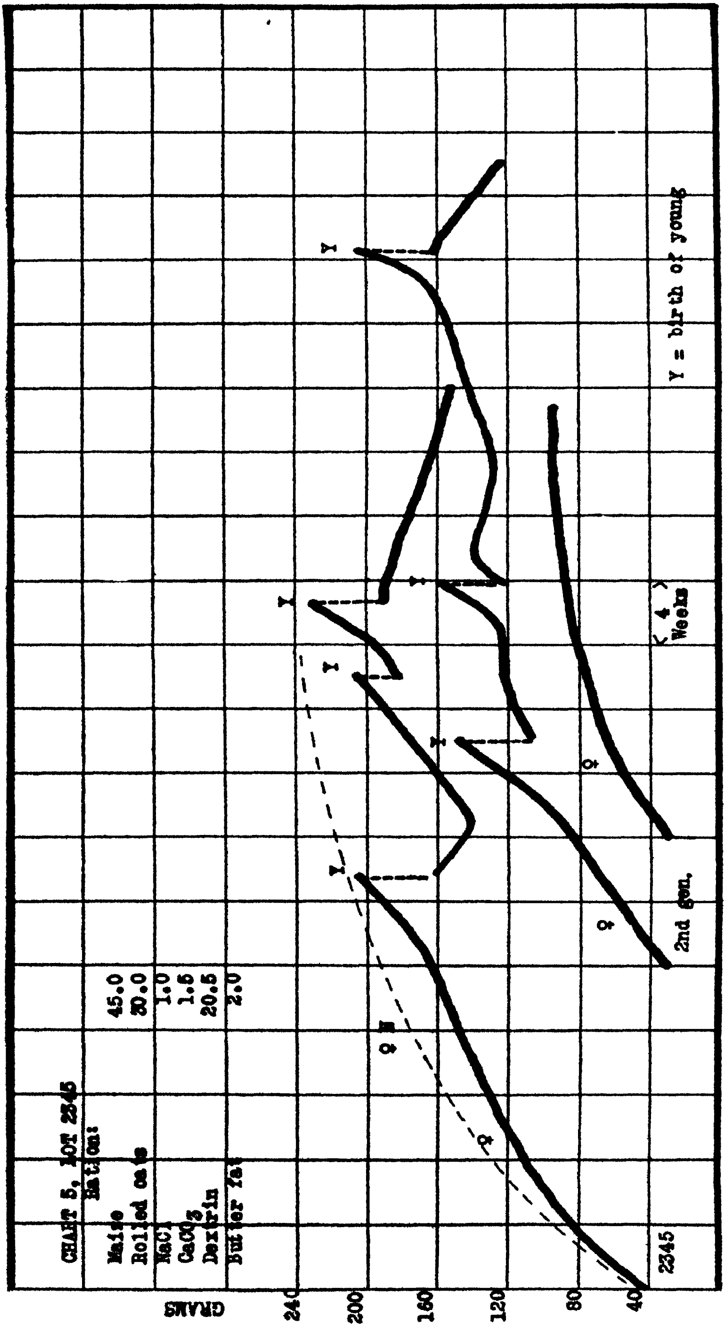


CHART 5, LOT 2345
Ration:
Maize 45.0
Rolled oats 30.0
NaCl 1.0
CaCO3 1.5
Dextrin 20.5
Butter fat 2.0
GRAMS
240
200
160
120
80
40
2345
♀
♀ M
2nd gen.
♀
♀
Y
Y
Y
Y
Y
Y
Y
< 4 > Weeks
Y = birth of young

Charts 1 to 5 make it clear that there is very little improvement effected in the quality of the proteins by combining two cereal grains or two legume seeds. This is so definite and striking that it forms an important contribution to the solution of the practical problems of nutrition. In Charts 6 to 13 it will be shown that in certain cases marked enhancement of the quality of the proteins is effected by making suitable combinations of a cereal grain with a legume seed.

Chart 6.—Lot 2381 derived its proteins (9 per cent of the food mixture) from barley (6 per cent) and soy beans (3 per cent). The growth curves were normal, and were therefore decidedly superior to any which we have secured with similar diets containing 9 per cent of barley proteins. 9 per cent of soy bean proteins induce but little growth when fed as the sole source of nitrogen (Chart 1, Lot 2510). These animals were still vigorous at 12 months but looked old at 17 months of age.

There were two females in the group. One was sterile, but the other had sixteen young (four litters), but did not wean any of them.

Lot 2364 was fed 9 per cent of protein derived from rolled oats (6 per cent) and navy beans (3 per cent). They remained undersized but their condition was better than that of rats fed a similar diet with but 9 per cent of oat protein as the sole source of nitrogen.

There were two females in the group. One died after 4½ months on the diet. The other had sixteen young (three litters) and weaned twelve. Six second generation females had thirty-eight young (seven litters) and weaned sixteen. Two third generation females were kept on the diet 4½ months but did not have any young. The nursing periods were rather long. A litter of five young, still with the mother, at 56 days weighed 215 gm., and were in fair condition.

Chart 7.—Lot 2378 had a diet containing 9 per cent of protein. 6 per cent was derived from wheat and 3 per cent from soy beans. The growth curves were normal, showing that there is an excellent supplementary relation between the proteins of these two seeds. This illustrates, when compared with other records of animals fed 9 per cent of proteins from various sources, many examples of which are shown in these charts, the great physiological advantage

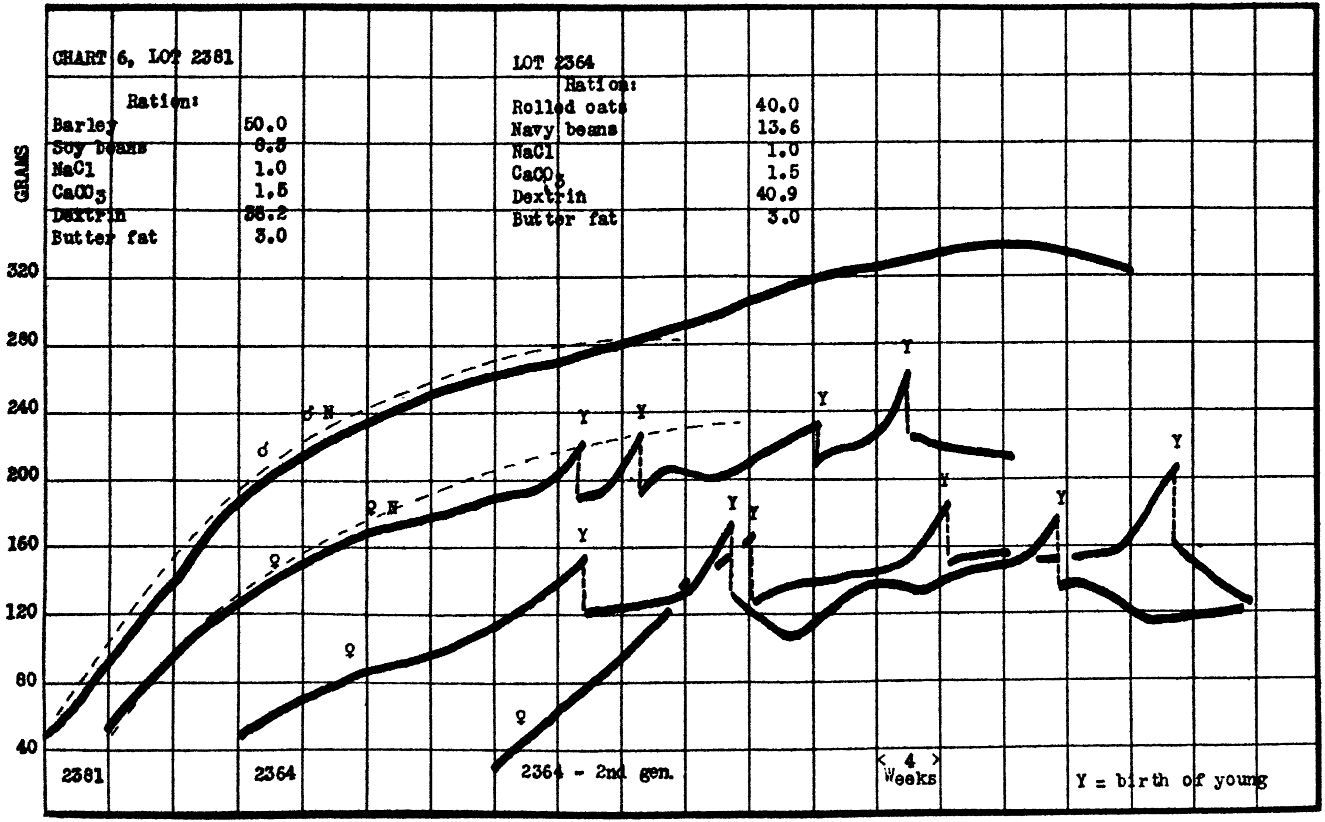


CHART 6, LOT 2381
Ration:
Barley 50.0
Soy beans 6.3
NaCl 1.0
CaCO3 1.5
Dextrin 38.2
Butter fat 3.0
LOT 2364
Ration:
Rolled oats 40.0
Navy beans 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 40.9
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
♂ N
♂
♀ N
♀
♀
♀
Y
2381
2364
2364 - 2nd gen.
< 4 >
Weeks
Y = birth of young

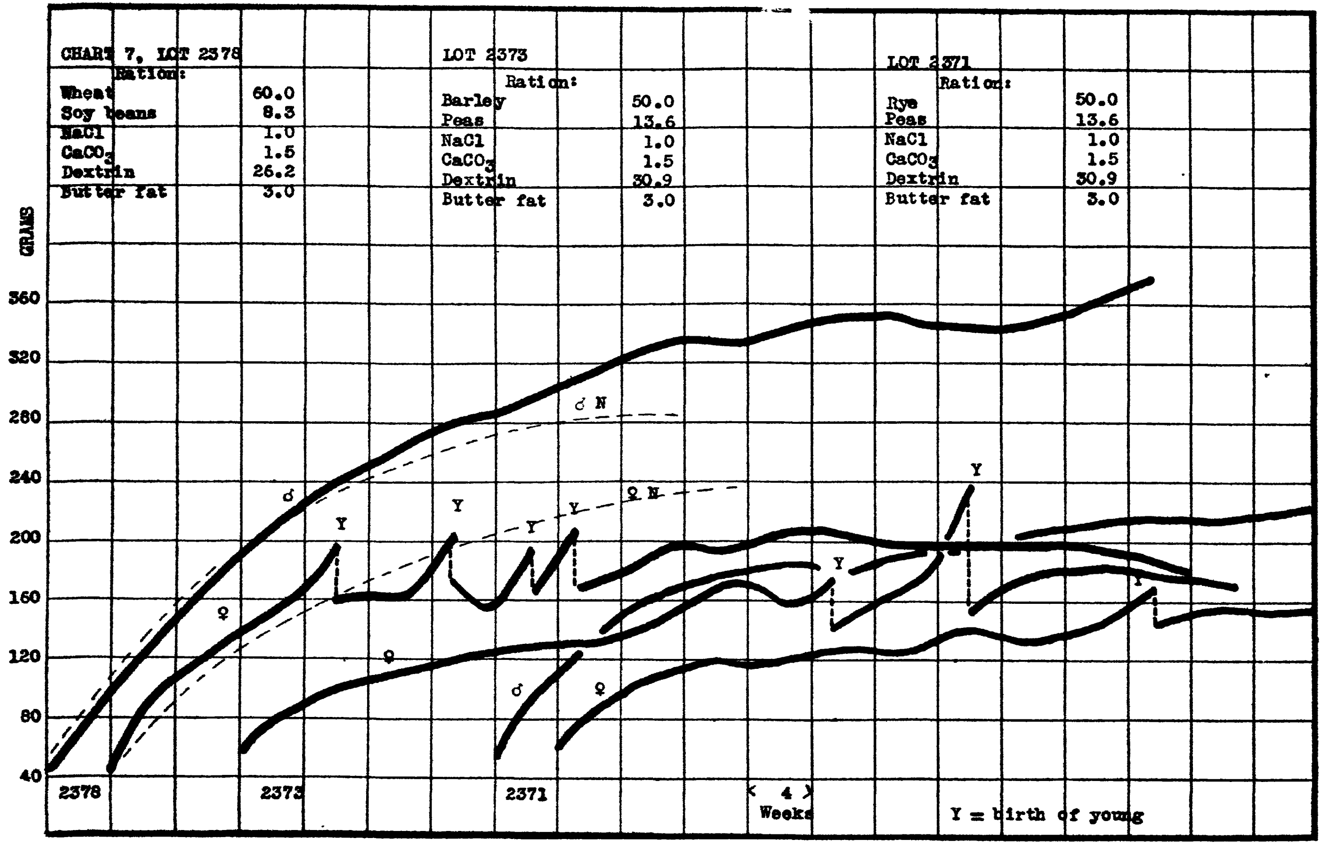


CHART 7, LOT 2378
Ration:
Wheat 60.0
Soy beans 8.3
NaCl 1.0
CaCO3 1.5
Dextrin 26.2
Butter fat 3.0
LOT 2373
Ration:
Barley 50.0
Peas 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 30.9
Butter fat 3.0
LOT 2371
Ration:
Rye 50.0
Peas 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 30.9
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
2378
2373
2371
< 4 >
Weeks
Y = birth of young
♂ N
♀ N
♂
♀
Y

to be derived from making appropriate combinations of natural foods so as to obtain protein mixtures having high biological values.

Two females had thirty-eight young (six litters) but destroyed them all soon after birth.

Lot 2373 derived its protein supply (9 per cent) from barley and peas. The barley supplied 6 per cent and the peas 3 per cent. These animals did not grow normally and were inferior in appearance, neglectful of their young, and were old looking at 17 months.

Three females had twenty-one young (six litters) and weaned but four. Two females of the second generation were put with the family group but were killed by the others.

Lot 2371 was fed 9 per cent of protein, 6 per cent from rye and 3 per cent from peas. The animals remained in a decidedly undersized condition and had very low fertility. One female was sterile. Another had one litter of young but destroyed them soon after birth.

Chart 8.—Lot 2365 derived its 9 per cent of protein from barley (6 per cent) and navy beans (3 per cent). The growth curves were good but the animals never reached the maximum size, nor were they in first class condition. They were very senile at 18 months of age.

Two females had thirty-three young (seven litters) and weaned eighteen, but the young were runty. They were always small for their ages and the nursing period was long. A litter of six weighed but 75 gm. when 24 days old. At 39 days five of these weighed 118 gm. At 59 days the five weighed 168 gm. This is less than one-third what they should have weighed.

Lot 2377 was fed maize equivalent to 6 per cent and soy beans equivalent to 3 per cent of the protein. The growth curves were better than can be secured with 9 per cent of maize proteins alone or with a similar amount of soy bean protein.

The females in this group were sterile. One died at 12 months, and the other at 14 months. Both were very old looking at these ages.

Chart 9.—Lot 2380 derived its protein, which constituted 9 per cent of the diet, from rolled oats (6 per cent) and soy beans (3 per cent). The growth curves do not indicate any appreciable supplementary relationship between the proteins of these two seeds.

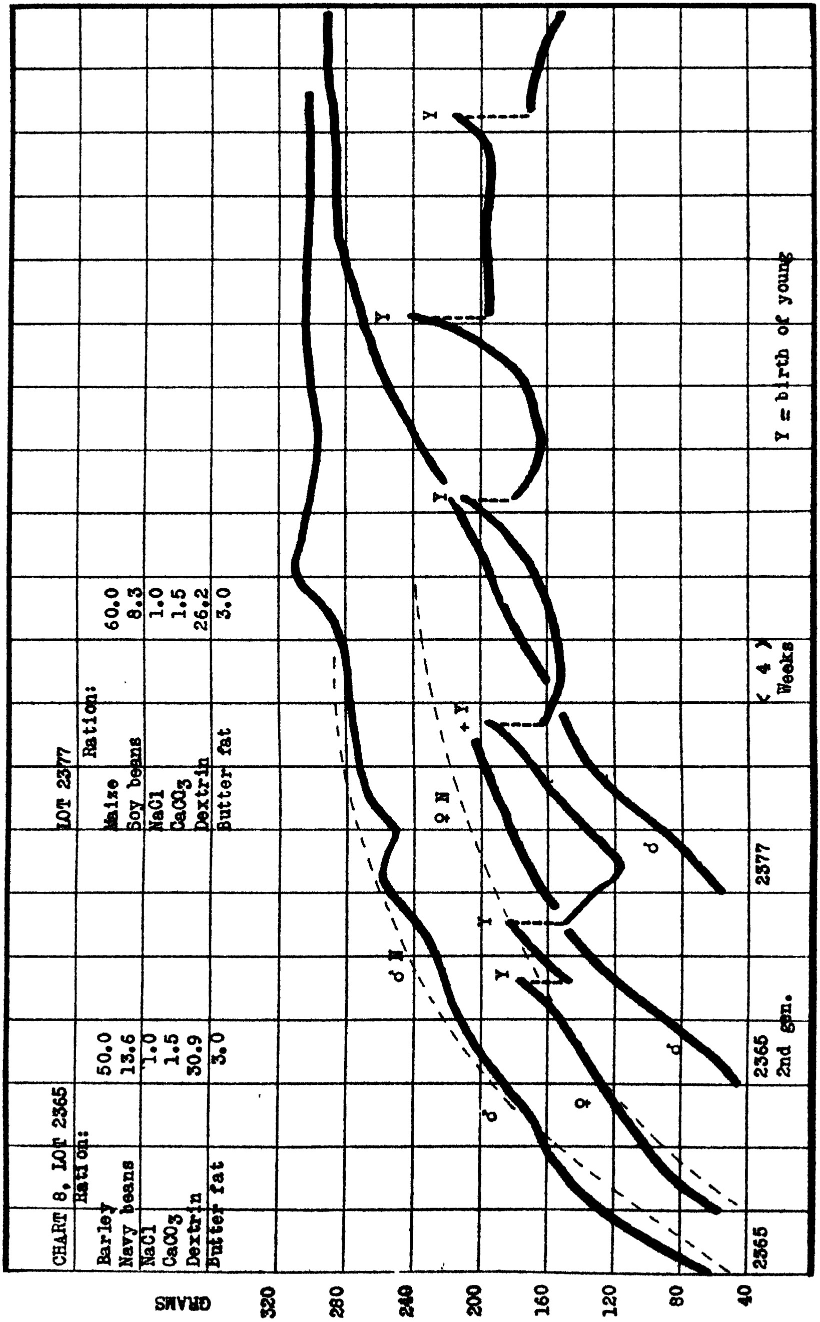


CHART 8. LOT 2365
Ration:
Barley 50.0
Navy beans 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 30.9
Butter fat 3.0
LOT 2377
Ration:
Maize 60.0
Soy beans 8.3
NaCl 1.0
CaCO3 1.5
Dextrin 26.2
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
2365
2365
2nd gen.
2577
< 4 >
Weeks
Y = birth of young
♂ N
♀ N
♂
♀
♂
♂
Y
Y
Y
Y
Y
Y

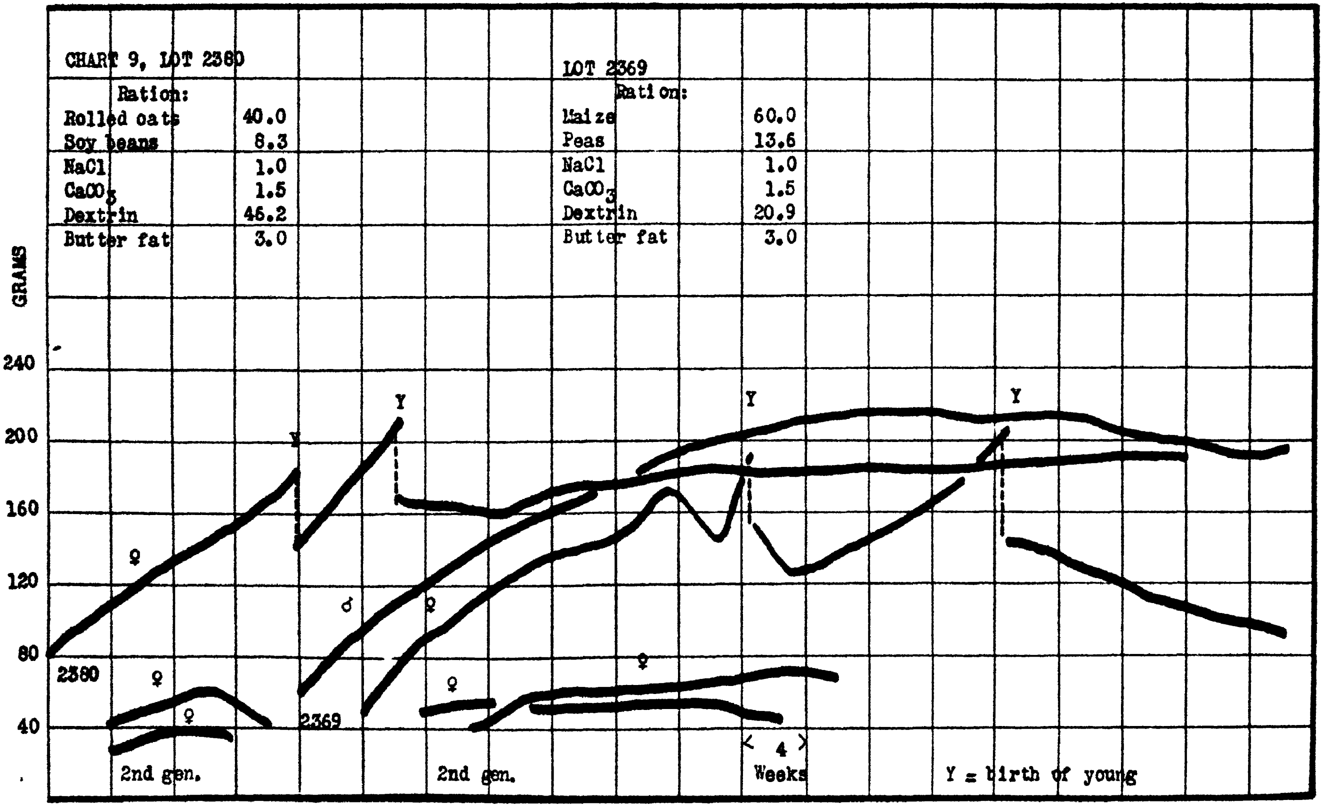


CHART 9, LOT 2380
Ration:
Rolled oats 40.0
Soy beans 8.3
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 46.2
Butter fat 3.0
LOT 2369
Ration:
Maize 60.0
Peas 13.6
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 20.9
Butter fat 3.0
GRAMS
240
200
160
120
80
40
2380
2369
♀
♂
Y
2nd gen.
2nd gen.
< 4 >
Weeks
Y = birth of young

Three females were confined to this diet. One never had any young. The other two had collectively fourteen young (three litters) and weaned three, which were always undersized and puny. A litter of seven was reduced by the mother to four at 16 days. At this age the four weighed 45 gm. At 35 days of age the four weighed 105 gm. and were not in a satisfactory condition. At 51 days but three survived. These weighed 102 gm. or less than half the size normal for young of this age.

Two second generation females were confined to the diet but never grew more than a few grams and died in 2 to 3 months after weaning.

Lot 2369 was fed 9 per cent of protein, two-thirds of which was furnished by maize and one-third by peas. Growth was distinctly below normal and was very inferior to a similar combination of wheat and peas (Chart 13, Lot 2370). The animals were always undersized and were old looking at 10 months and very ragged looking at 12 months.

Two females grew up on the diet. One remained sterile. The other had fourteen young (two litters) and weaned ten. The young were runty, and required a long nursing period. A litter of six young was reduced by the mother in 7 days to four. These weighed at this time 37 gm. At 31 days the four weighed 107 gm. At 46 days the four weighed 123 gm. Two females in the second generation remained undersized and never had any young.

Chart 10.—Lot 2372 derived its 9 per cent of protein from rolled oats (6 per cent) and peas (3 per cent). The growth curves were somewhat below the maximum, but much better than could be secured from 9 per cent of protein from oats or peas alone. There is, therefore, an excellent supplementary relationship between the proteins of the oat and pea, but this is not so effective as is a combination of proteins of wheat and peas (Chart 13, Lot 2370). Oat and pea proteins are better than maize and peas, as is shown by a comparison with Lot 2380 (Chart 9).

There were two females in this group. One had twenty-five young (three litters) and weaned thirteen. The young were never vigorous. One female died at 6 months from an unknown cause. One second generation female had ten young (three litters) but destroyed them soon after birth. The nursing periods were long

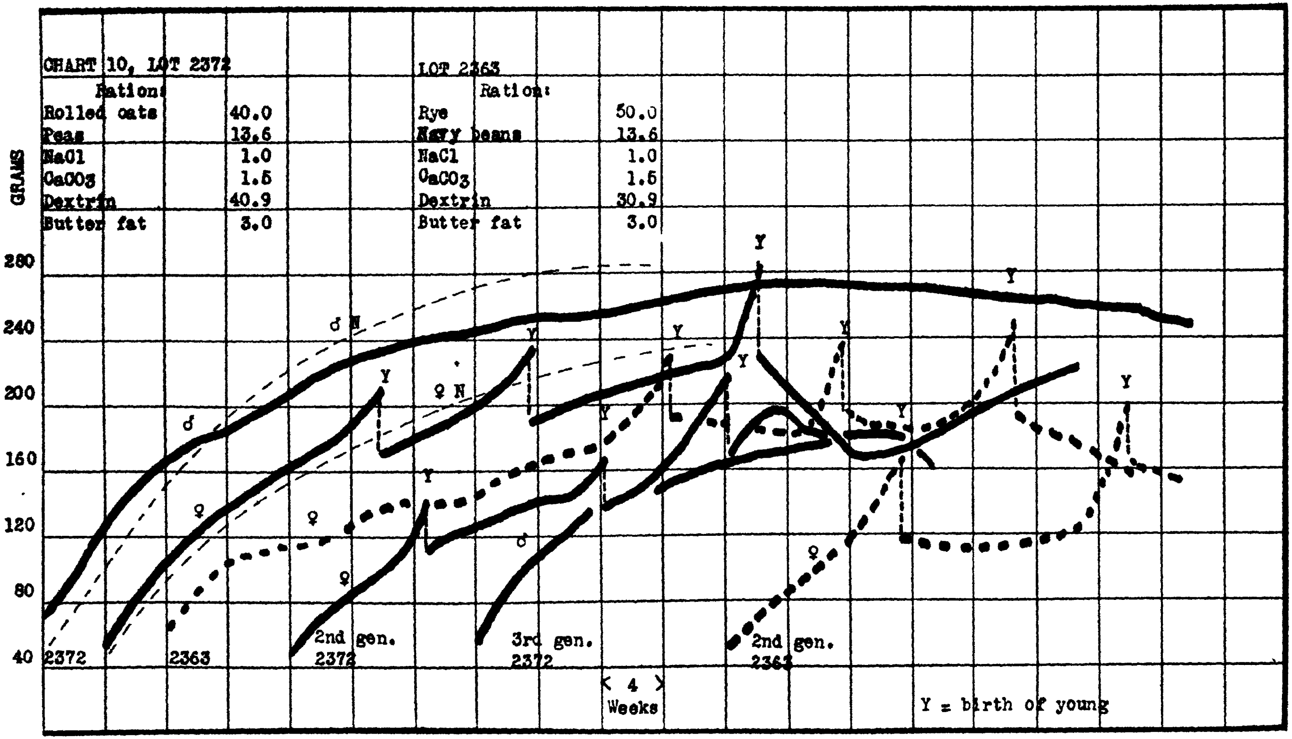


CHART 10, LOT 2372
Ration:
Rolled oats 40.0
Peas 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 40.9
Butter fat 3.0
LOT 2363
Ration:
Rye 50.0
Navy beans 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 30.9
Butter fat 3.0
GRAMS
280
240
200
160
120
80
40
2372
2363
2nd gen.
2372
3rd gen.
2372
2nd gen.
2363
♂
♀
♂ N
♀ N
Y
< 4 >
Weeks
Y = birth of young

in all cases. A litter of eight weighed 65 gm. at 14 days. At 22 days the mother had reduced the number to six, which weighed 55 gm. At 48 days there were but five remaining. These weighed 111 gm. At 76 days but three remained. These weighed 107 gm.

Lot 2363 was fed 9 per cent of protein, 6 per cent being derived from rye and 3 per cent from navy beans. The growth records and fertility were decidedly below normal. The nursing periods were greatly prolonged and the infant mortality was high. After being restricted to the diet for 18 months the animals looked very old.

Two females had collectively thirty-one young (five litters) and weaned twenty-two. A litter of nine young was reduced by the mother to eight by the 11th day. These weighed 59 gm. They were reduced to five by 34 days, and the litter weighed 80 gm. At 55 days the litter was reduced to three, weighing 85 gm. This is less than one-third the normal weight for this age. Two second generation females had twenty-three young (four litters). One third generation female had a single litter of two, but destroyed them soon after birth.

Chart 11.—Lot 2361 was fed maize and navy beans to furnish 9 per cent of protein in the diet. Maize furnished 6 per cent and navy beans 3 per cent of the protein. The animals grew slowly and remained somewhat undersized, their fertility was low, and infant mortality high. In Lot 814 we[7] have secured much better growth and reproduction on a diet containing 7.4 per cent of maize and 4.4 per cent of navy bean proteins, respectively. This is a remarkable illustration of the physiological effects which may result from a small variation in the amount of protein in the diet.

There were two females in this group (Lot 2361). They had fourteen young (three litters) and weaned eight. The young were small for their ages. One litter of seven was reduced by the mother to six at 17 days. These weighed but 60 gm. At 42 days the six weighed 145 gm. One second generation female was kept on the diet 11½ months but never had any young.

Lot 2379 derived the 9 per cent of protein in its diet from rye (6 per cent) and soy beans (3 per cent). The animals remained somewhat undersized but appeared to be in good condition.

Two females had forty-three young (five litters) and weaned fifteen. The young were never in good condition and the nursing

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[7] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 54.

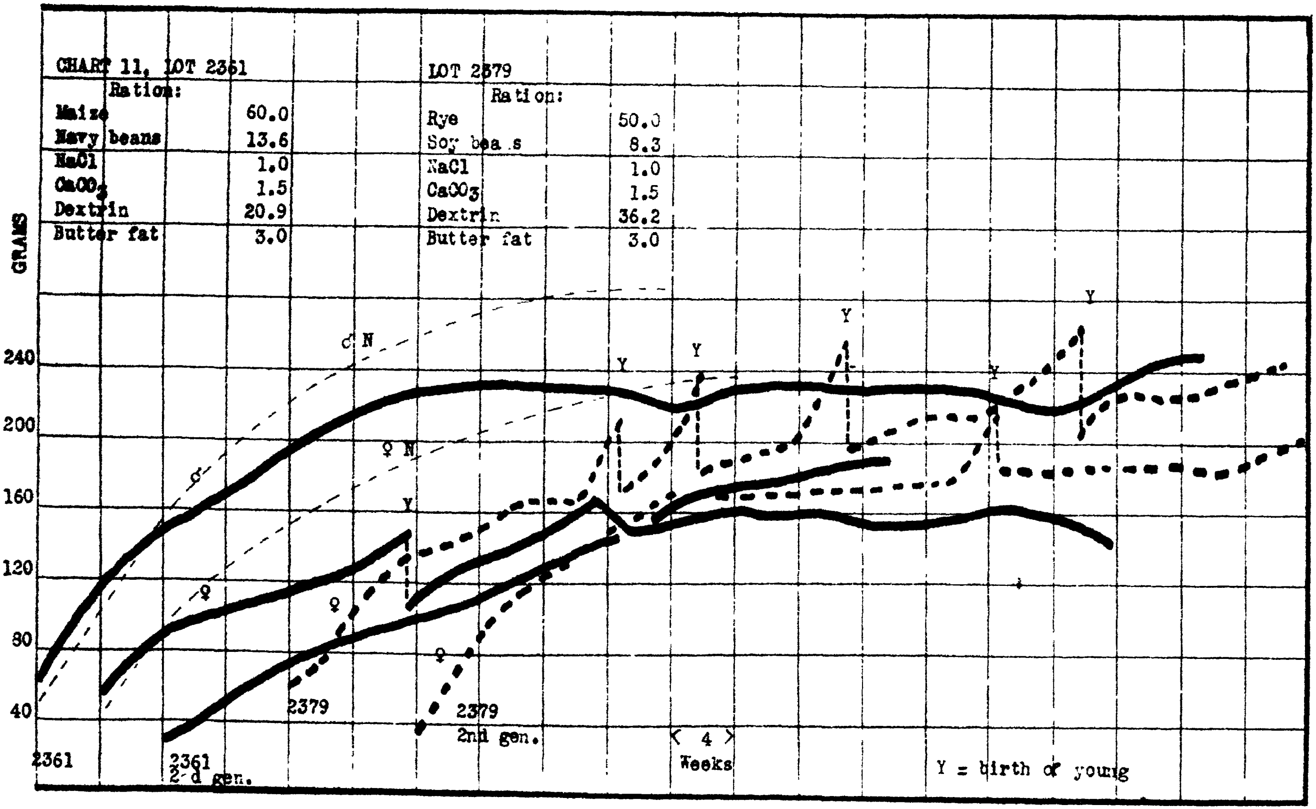
CHART 11, LOT 2361

Ration:	
Maize	60.0
Navy beans	13.6
NaCl	1.0
$CaCO_3$	1.5
Dextrin	20.9
Butter fat	3.0

LOT 2379

Ration:	
Rye	50.0
Soy beans	8.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	36.2
Butter fat	3.0

period was long. Three second generation females grew up on the diet. Two of these remained sterile. The other had a litter of four and weaned one.

One of the females of the original group had a litter of eleven. At 8 days these weighed collectively 85 gm. By 20 days they were reduced by the mother to ten. These weighed 110 gm. On the 35th day five of these died. On the 41st day but two were left. These weighed but 37 gm. This was less than one-third what they should have weighed. These two remaining ones were killed and eaten by the mother at 51 days. The history of this litter of young is typical of many we have seen in groups of animals restricted to diets which were satisfactory in every respect except in quality or amount of protein.

Chart 12.—Lot 2362 took a diet containing 9 per cent of protein derived from wheat (6 per cent) and navy beans (3 per cent). The growth records and fertility were good but the infant mortality was high.

Two females had thirty-seven young (nine litters) and weaned eleven. Many young were destroyed by the mothers soon after birth. Two second generation females had thirty-five young (nine litters) and weaned seventeen. One second generation female remained sterile. The young were always inferior in size, appearance, and vigor.

Chart 13.—Lot 2370 derived its 9 per cent of protein from wheat (6 per cent) and peas (3 per cent). The growth curves of this group were the best we have observed in rats fed but 9 per cent of protein derived from a combination of vegetable proteins from two sources. The animals reached full adult size, the fertility was high, and the success in rearing young was better than in any other group described in this series, or in any instance in our experience where rats were grown and maintained on a diet containing but 9 per cent of *vegetable* proteins.

Two females had forty-nine young (nine litters) and weaned thirty-four. Two second generation females were grown on the family diet. One remained sterile. The other had twenty-six young (four litters) and weaned twenty-one. One third generation female had ten young (two litters) and weaned eight. The young were always somewhat small for their ages and the nursing periods rather prolonged because of the limitation placed upon the mothers by the low protein content of the diet.

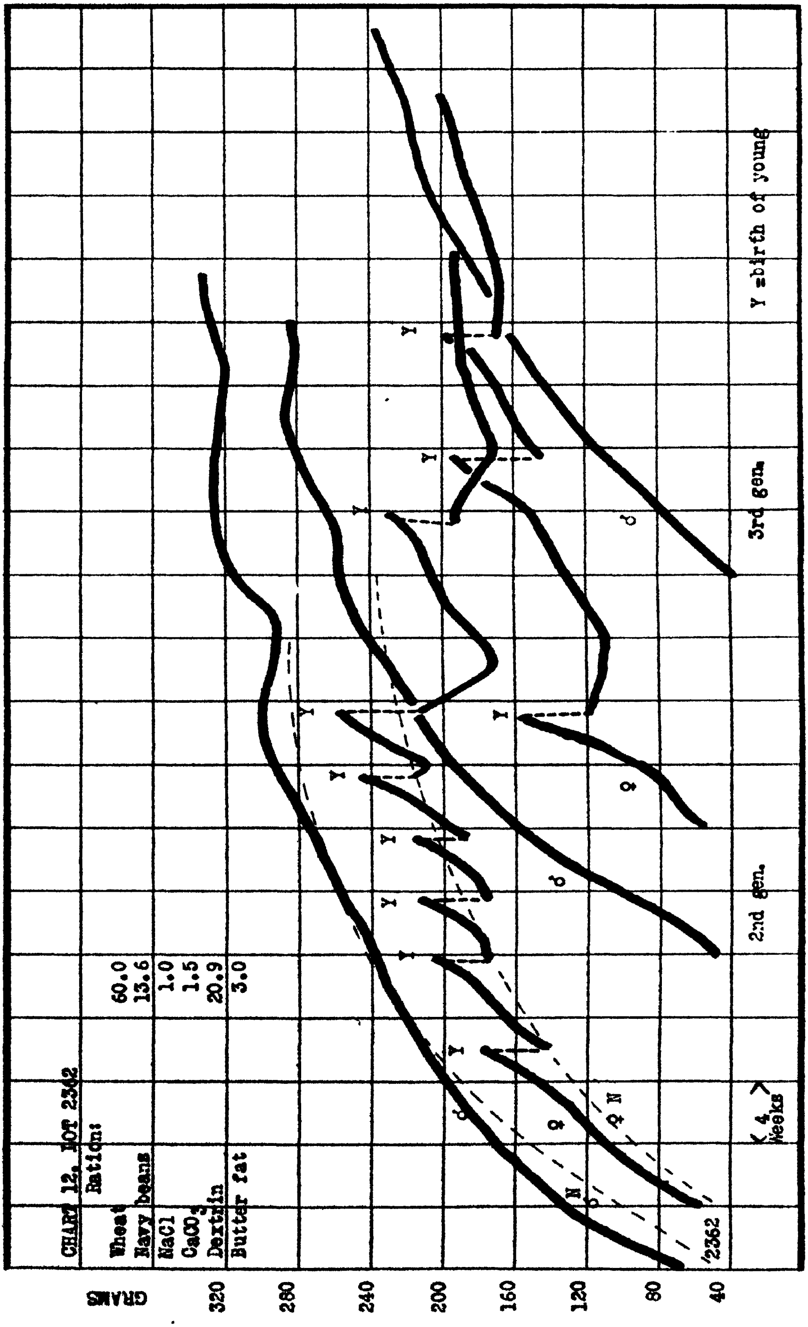
CHART 12. LOT 2362
Ration:
Wheat 60.0
Navy beans 13.6
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 20.9
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
Y = birth of young
3rd gen.
2nd gen.
Y
♂
♀
N
♀ N
2362
< 4 >
Weeks

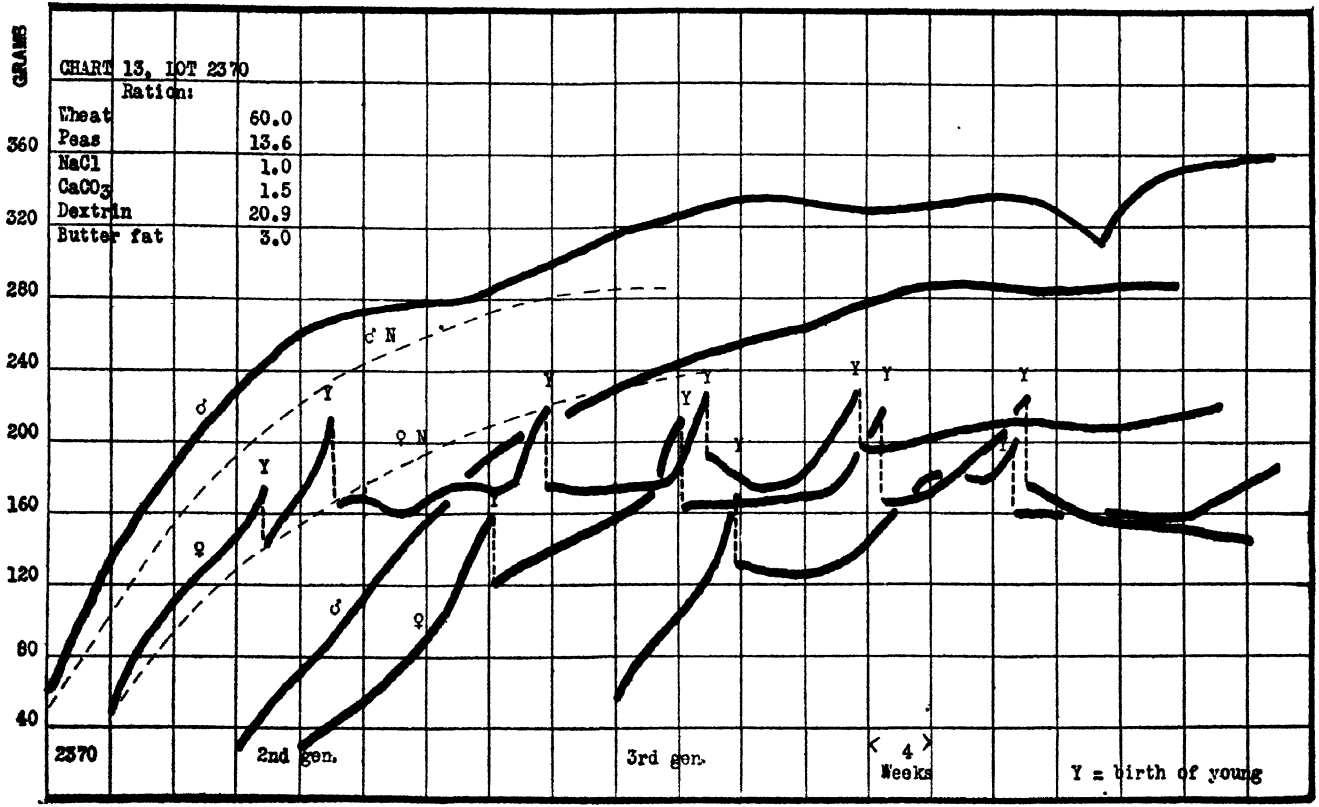
CHART 13, LOT 2370
Ration:
Wheat 60.0
Peas 13.6
NaCl 1.0
CaCO3 1.5
Dextrin 20.9
Butter fat 3.0
GRAMS
360
320
280
240
200
160
120
80
40
♂ N
♀ N
♂
♀
♂
♀
Y
2370
2nd gen.
3rd gen.
< 4 >
Weeks
Y = birth of young

SUPPLEMENTARY PROTEIN VALUES IN FOODS.

V. SUPPLEMENTARY RELATIONS OF THE PROTEINS OF MILK FOR THOSE OF CEREALS AND OF MILK FOR THOSE OF LEGUME SEEDS.

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(Received for publication, March 21, 1921.)

The nature of the dietary deficiencies of the cereal and legume seeds is now well understood.[1] It has also been shown that milk and the leafy vegetables occupy a unique place among our ordinary foodstuffs in that they are the only foods regularly consumed in moderate quantities which are of a nature to correct the mineral deficiencies of cereals, legume seeds, tubers, and fleshy roots, or to adequately supplement them with respect to fat-soluble A. It is well known from laboratory experiments on animals as well as from agricultural experience, that milk proteins tend to enhance the value of vegetable proteins generally.[2] Specific information as to the extent to which milk proteins supplement those of individual vegetable foods is still wanting. The studies reported in this paper form a contribution to this phase of our knowledge of practical dietetics.

In these experiments the proteins of the diets were derived from half skimmed milk powder (Merrell-Soule) and a single plant seed or tuber. In order to bring out more clearly the extent of the supplementary relations between the proteins employed we have in all cases limited the content of this factor to 9 per cent of the food mixture. We have already pointed out that in order to secure a normal growth curve in the rat on this low plane of protein intake, the quality of protein for growth must be good.[3] When the growth curves fall below normal the extent of retardation of development serves as a good index to the extent to which the quality of any mixture under investigation falls below that of the best combinations which we have been able to discover. When, in addition, we observe the animals throughout their reproductive period and secure records of fertility and infant mortality, and the time at which the first signs of old age appear, we have the most sensitive indexes to physiological well being which it seems probable will ever be observable. Proteins of good quality will induce normal growth when fed at the plane of intake of 9 per cent of the food mixture, and may induce a fair degree of fertility. In order to secure high fertility and low infant mortality the proteins must be of excellent quality.

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[1] McCollum, E. V., The newer knowledge of nutrition, New York, 1918.

[2] McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.

In a former paper[4] we have described comparable studies with diets in which the proteins were derived from combinations of either liver, kidney, or muscle with cereal or legume seed proteins. The plane of protein intake was uniform (9 per cent) in all cases. These records, together with those presented in the present paper, form, therefore, a contrast between the value of milk proteins on the one hand and animal tissue proteins on the other, as supplements for a number of vegetable foods with respect to the protein moiety.

The results bring out in a very striking way the unexpected superiority of animal tissue proteins for the special purpose of enhancing the value of various plant seed proteins. In our numerous studies of this phase of nutrition we have demonstrated that with diets of the type containing 9 per cent of protein, and with all other factors satisfactorily adjusted, the proteins of kidney produce the best results we have yet observed. The biological value of the proteins of animal tissues for growth or for maintenance of health differs in an easily demonstrable degree.

An inspection of the results of our experiments with diets of the type here employed warrants arranging the proteins of a number of animal and vegetable foods of great importance in a series showing their relative nutritive values. In the following scheme we have arranged the several foods in order of the biological value of their proteins. The best is placed at the left and the series is a descending one.

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[3] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 155.

[4] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1921, xlvii, 139.

$$\text{Beef kidney—Wheat—}\left\{\begin{array}{l}\text{Milk}\\ \text{Liver—}\\ \quad\text{(beef)}\end{array}\right.\left\{\begin{array}{l}\text{Muscle}\\ \quad\text{(round steak)}\\ \text{Barley}\\ \text{Rye}\end{array}\right\}\text{—}\left\{\begin{array}{l}\text{Maize}\\ \text{Oats}\end{array}\right.\text{—}\left\{\begin{array}{l}\text{Soy beans}\\ \text{Navy beans}\\ \text{Pea}\end{array}\right.$$

In our earlier studies of the cereal grains[5] we became convinced that wheat proteins were of somewhat lower biological value than more recent experimental data would seem to indicate. This may perhaps be accounted for by differences in the proteins of various samples of wheat. It is well known that the dough-forming quality varies markedly in wheats. This property depends upon the peculiar nature of the proteins of this grain and may be due to lack of uniformity in the relative amounts of the individual proteins contained in the seed.

It should be kept in mind that such a differentiation in biological value of proteins from these foods will not be apparent unless the experimental procedure is appropriate to bring them out. The several foods included in the scheme must be fed, with other dietary factors satisfactorily constituted, at such a plane of intake as to furnish the critical level of 9 per cent of protein in the diet. This is the only method we have been able to devise to show these differences. The observations must include not only the period of growth but also the fertility, the success with young, and the period following the completion of growth to the point where senile characters are apparent.

We were surprised to find how consistently combinations of milk proteins and cereal or legume proteins fail to show as high biological values as can be demonstrated for kidney, liver, and muscle proteins combined with those of certain cereals.[4] It should not be lost sight of that milk has an effective supplementary relation to cereals both with respect to the inorganic and fat-soluble A deficiencies of the latter, whereas muscle meats supplement them only with respect to the protein factor, and glandular organs only with respect to protein and fat-soluble A. This fact is brought out in a striking way by the records in Chart 5. When pasteurized or boiled milk, or cooked glandular organs are contained in the diet they do not effectively supplement a cereal and legume seed mixture with respect to the antiscorbutic factor since this is a labile substance. This factor is essential in the nutrition of man, monkey, and guinea pig, but need not be furnished by the diet of the rat or prairie dog, since they are apparently able to synthesize it.[6]

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[5] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916–17, xxviii, 211. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

SUMMARY.

We have described in this and preceding papers experiments which were so planned as to compare the relative merits of animal tissue proteins and of milk proteins for enhancing the value of the proteins of each of the following vegetable foods: barley, peas, soy beans, rye, maize, navy beans, wheat, potato, and rolled oats. These indicate clearly that animal tissues such as liver, kidney, or muscle, are superior to milk for the specific purpose of making good the deficiencies of the proteins of the seeds and tuber mentioned above.

Milk, however, is an effective supplement for these vegetable foods with respect to other factors as well as protein. This is especially true of calcium and fat-soluble A.

In making deductions from these results it should be kept in mind that muscle tissue supplements seeds, tubers, etc., only with respect to the protein factor, and that other deficiencies of even greater importance for the well being of the body are always met with in that group of vegetable foods which are functionally storage tissues of plants; *viz.*, seeds, tubers, and roots.

Chart 1.—Lot 2391 was fed a diet containing 9 per cent of protein derived from barley (6 per cent) and milk (3 per cent). All other factors in the diet were made satisfactory by suitable additions of inorganic elements and fat-soluble A (in butter fat). Growth took place at a subnormal rate and the full adult size was never attained. The animals in this group looked rough coated and old at 15 months.

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[6] McCollum, E. V., and Parsons, H. T., *J. Biol. Chem.*, 1920, xliv, 603.

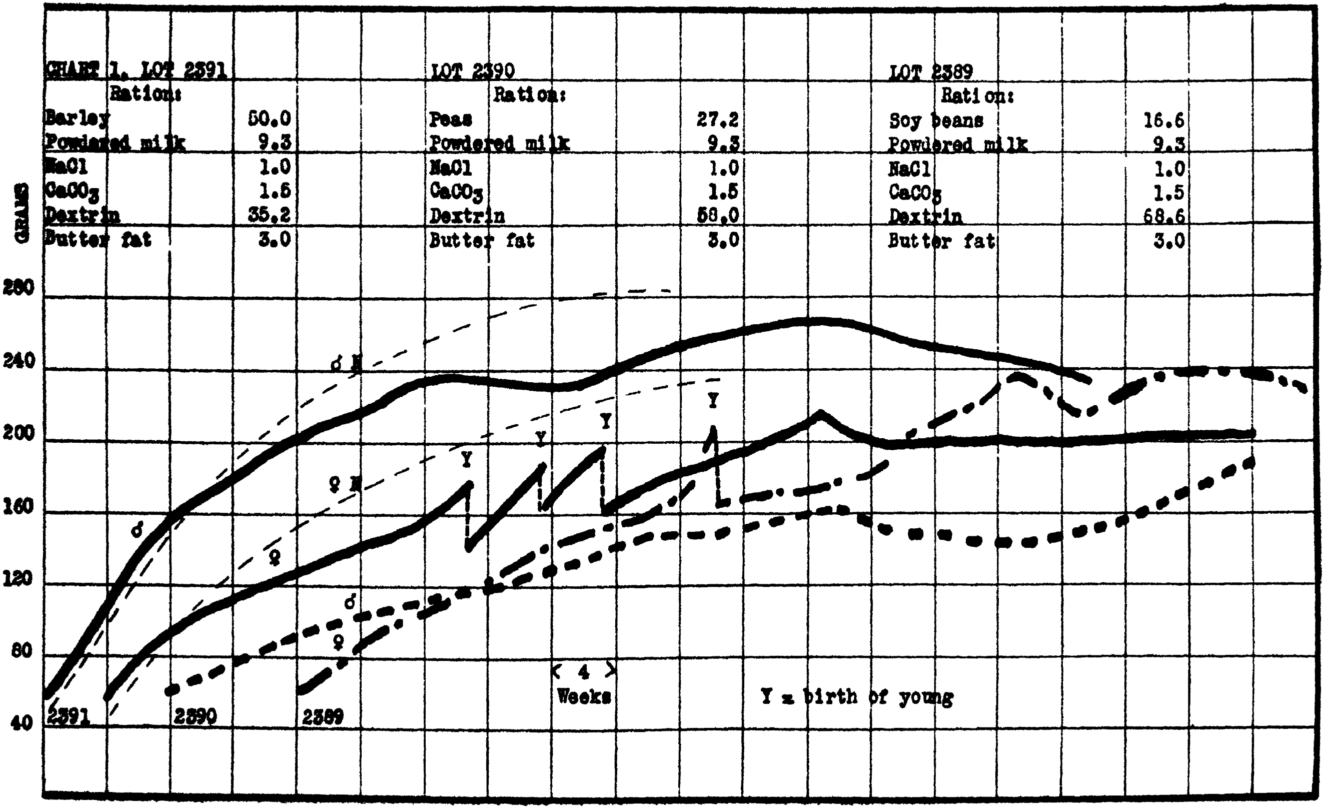


CHART 1. LOT 2391

Ration:	
Barley	50.0
Powdered milk	9.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	35.2
Butter fat	3.0

LOT 2390

Ration:	
Peas	27.2
Powdered milk	9.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	58.0
Butter fat	3.0

LOT 2389

Ration:	
Soy beans	16.6
Powdered milk	9.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	68.6
Butter fat	3.0

Two females grew up on this diet. One remained sterile. The other had eleven young (three litters) but destroyed them soon after birth.

Lot 2390 derived its diet, containing 9 per cent of protein, from peas (6 per cent) and powdered milk (3 per cent). The protein of the diet was the limiting factor. The animals all grew poorly, but better than they could have grown on 9 per cent of pea protein alone. These rats were very apprehensive and could be weighed only with difficulty because of constant efforts to escape. They were so excited when handled that they would not sit still in a a small covered box but would constantly spring up and strike the lid with their heads. The hair was very short and fine, and had a silky appearance, which we have never seen on rats fed highly satisfactory diets. The same type of coat has been frequently met with in rats restricted in a great measure to maize as a source of protein. Less frequently we have seen these "mole-skin" rats in groups which derived their protein from kafir corn.

Two females were restricted to this diet but never had any young.

Lot 2389 was fed soy beans and powdered milk as a source of protein. The total protein content of the diet was 9 per cent. The soy beans furnished two-thirds and the milk one-third of the total. While the growth was much better than we have ever seen on 9 per cent of soy bean protein alone, the rate of growth was distinctly below normal. The animals remained undersized, and their fertility was very low. There were three females in this group. One had a litter of seven. At 14 days they weighed 57 gm. At 21 days one had died. The remaining six weighed 62 gm. At 27 days but four survived and these weighed collectively but 45 gm. They were very puny and incapable of growing on the mother's milk. The other two females remained sterile.

Chart 2.—The rats of Lot 2386 were fed 9 per cent of protein derived from rye (6 per cent) and milk powder (3 per cent). Growth was slow but the animals reached nearly the adult size after some delay. These rats aged decidedly early. They looked old at 14 months. The second generation were all more undersized than the first. We have observed in many cases where the food mixture was faulty to a slight degree and a family was con-

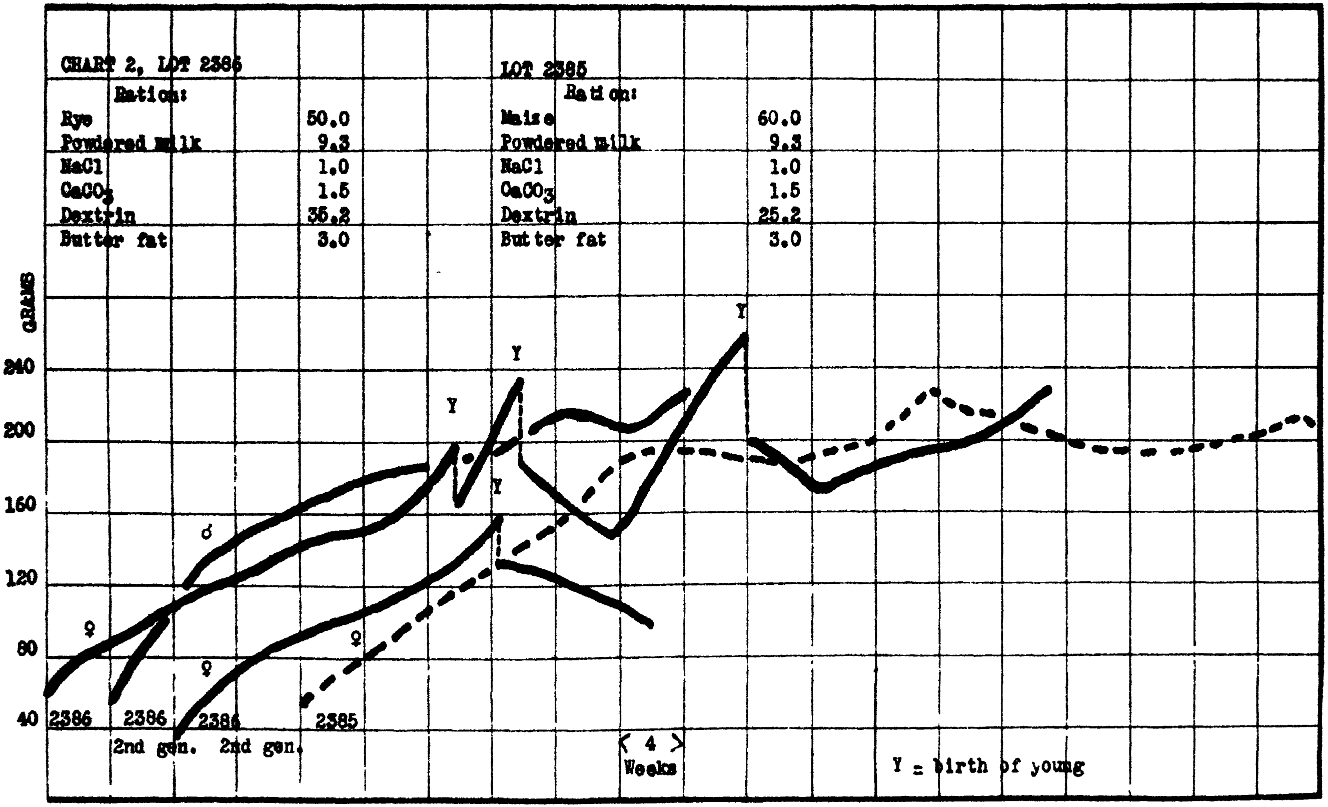


CHART 2, LOT 2386

Ration:	
Rye	50.0
Powdered milk	9.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	35.2
Butter fat	3.0

LOT 2385

Ration:	
Maize	60.0
Powdered milk	9.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	25.2
Butter fat	3.0

Y = birth of young

fined to the diet through several generations, each succeeding generation was smaller than the preceding one when growth was completed.

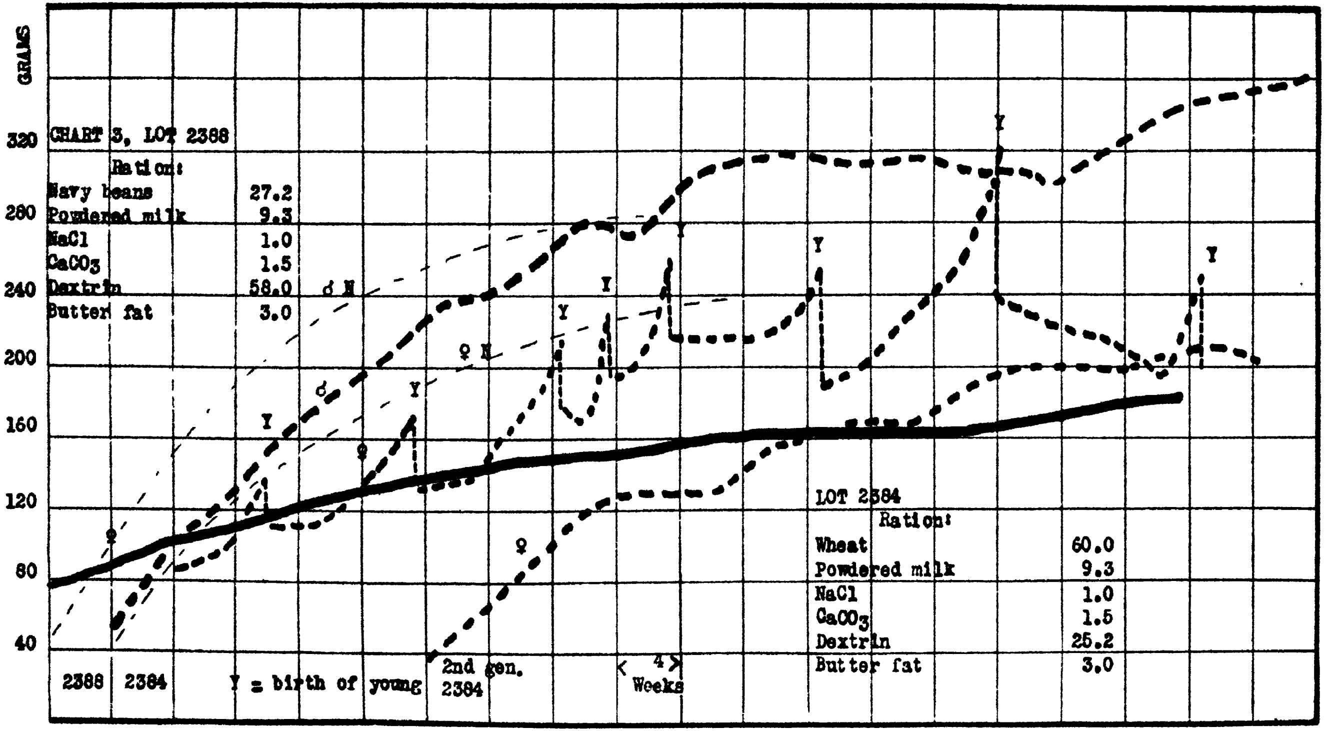
There were three females in the group. Two of these remained sterile. The other had twenty-eight young (three litters) and weaned eleven. The nursing period was, however, very long. A litter of eight was reduced by death to five by the 32nd day. These weighed 108 gm. At 39 days they weighed 121 gm., and were in a poorly developed condition. They were less than one-third the normal size for their age. One second generation female had a single litter but destroyed it soon after birth.

Lot 2385 was fed 9 per cent of protein derived from maize (6 per cent) and milk powder (3 per cent). All other factors in the diet were made satisfactory by suitable additions. Growth was slow but the animals reached nearly the full adult size. There were three females in the group but none ever had any young. The hair of this group was short and silky and suggestive of a mole skin.

Chart 3.—Lot 2388 derived the 9 per cent of protein in its diet from navy beans (6 per cent) and milk powder (3 per cent). The animals grew slowly and remained permanently undersized. They lived surprisingly long on this diet on which they grew so poorly. The history of the group on 9 per cent of protein from navy beans and milk is comparable to that of Lot 2390 (Chart 1), which was identical except that peas replaced the beans. The same diet with soy beans in place of peas or navy beans produced distinctly better growth (Lot 2389, Chart 1).

Lot 2384 derived the 9 per cent protein in its diet from wheat (6 per cent) and milk powder (3 per cent). The combination of wheat and milk proteins is better than a similar amount of protein from wheat alone. With the exception of the diet of oats and milk (Lot 2387, Chart 4), this food mixture was superior to any other combination of seed with milk proteins which we have studied. The animals appeared old after about 19 months on this diet.

There were two females in the group, one of which died after being 4½ months on the diet. The other had forty-one young (seven litters) and weaned nineteen. The nursing periods were long in all cases. The young were not destroyed in the ruthless


GRAMS
320
280
240
200
160
120
80
40
CHART 3, LOT 2388
Ration:
Navy beans 27.2
Powdered milk 9.3
NaCl 1.0
CaCO3 1.5
Dextrin 58.0
Butter fat 3.0
♂ N
♀ N
♂
♀
♀
♀
Y
Y
Y
Y
Y
Y
Y
Y
LOT 2384
Ration:
Wheat 60.0
Powdered milk 9.3
NaCl 1.0
CaCO3 1.5
Dextrin 25.2
Butter fat 3.0
2388
2384
Y = birth of young
2nd gen.
2384
< 4 >
Weeks

manner frequently observed, but died at intervals from undetermined causes.

A litter of five young weighed but 103 gm. at 23 days of age. At 58 days four weighed 141 gm. They appeared to be in good condition but were undersized. Two second generation females had but five young (one litter) and weaned one.

Chart 4.—Lot 2403 derived the 9 per cent of protein which its diet contained from potatoes (6 per cent) and milk powder (3 per cent). Growth was somewhat below normal and they remained undersized. These rats aged very early. They looked as old at 1 year as many better nourished animals do at 18 months.

Three females had twenty-seven young (five litters) and weaned only two. The nursing periods were long. A litter of five weighed 40 gm. at 15 days. At 26 days they were reduced by death to three, which weighed collectively 47 gm. At 60 days but two were left. These weighed together 70 gm. This is less than half what they should have weighed at this age.

The nitrogen of the potato is in great measure in the form of simple substances of a non-protein nature. These substances are evidently not of a character which supplements the proteins of milk to any marked extent. We have pointed out elsewhere that the nitrogen of the potato when fed as the sole source of this factor is not of so high a value as some have reported.[7]

Lot 2387 was fed 9 per cent of protein, two-thirds of which was derived from rolled oats and one-third from milk powder. This combination of proteins seems to have a higher value than any other cereal and milk mixture we have investigated. But little inferior to this is the wheat and milk combination. We have in some earlier experiments seen better curves of growth on about this amount (8 per cent) of protein from a mixture of oats and milk. After 16 months on the diet their coats (Lot 2387) were somewhat rough, but the animals were still vigorous.

Three females had thirty-three young (five litters) of which six were weaned. Three other litters were destroyed before their numbers could be determined. The nursing periods were long. A litter of seven young weighed 66 gm. at 15 days of age. At 34

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[7] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxvi, 197.

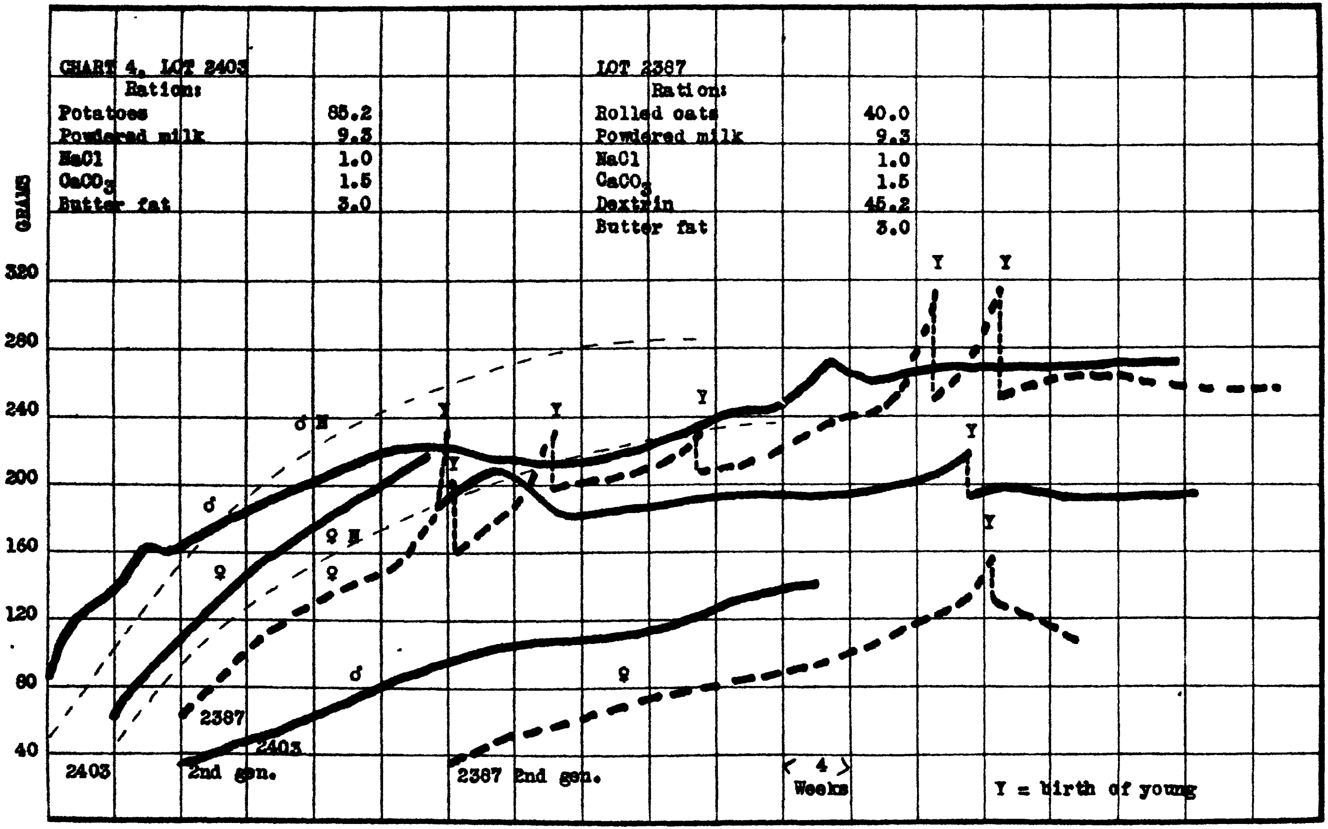
CHART 4. LOT 2403
Ration:
Potatoes 85.2
Powdered milk 9.3
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
LOT 2387
Ration:
Rolled oats 40.0
Powdered milk 9.3
NaCl 1.0
CaCO3 1.5
Dextrin 45.2
Butter fat 3.0
GRAMS
320
280
240
200
160
120
80
40
♂
♀
♂ N
♀ N
♀
2387
2403
2403
2nd gen.
♂
♀
2387 2nd gen.
Y
Y
Y
Y
Y
Y
Y
Y
< 4 >
Weeks
Y = birth of young

days they weighed collectively 107 gm. At 60 days six of these weighed 202 gm., or less than half the normal for this age. Two second generation females grew up on the diet. One was sterile. The other had one litter of three after she had been 8 months on the diet. She weaned them all after a long nursing period.

Chart 5.—Lots 2148 and 2147 show the relative merits of milk and of muscle tissue as supplements to a mixture of foodstuffs consisting of articles which are functionally storage organs of plants. The mixture, aside from the milk or muscle meat, consisted of two fleshy roots, red beet and yellow turnip; a tuber, the potato; two legume seeds, pea and navy bean; and two degerminated cereal products, wheat flour and corn-meal (maize).

Notwithstanding the wide variety in such a list of foods, and an appropriate chemical composition as indicated by the ordinary food analysis, it does not promote growth in young animals nor support the vitality of adults as measured by fertility, success in rearing young, or in deferring the onset of old age.

Muscle meat (round steak) supplements a mixture of cereals, legume seeds, fleshy roots, and tubers only with respect to the protein factor. Milk on the other hand enhances not only the protein of these vegetable foods, but likewise makes good their mineral deficiencies and also the shortage of fat-soluble A which all such mixtures exhibit.

Lot 2147 in which the vegetable diet of storage organs is supplemented only with muscle meat, failed to grow normally. The curve shown is typical of a group of six animals restricted to this diet. They never had any young and aged very early. They looked extremely rough coated after 6 to 8 months on the diet. Lot 2148, on the other hand, whose diet was similar in all respects but contained 10 per cent of whole milk powder, grew normally and remained in much better condition to an age of about 18 months. These animals showed fair fertility and success in rearing their young. The milk supplemented not only the proteins of vegetable origin but accomplished what was of greater importance; *viz.*, the correction of the inorganic deficiencies and made good in great measure the lack of fat-soluble A. The bearing of such observations as these on practical human dietetics will be easily appreciated.

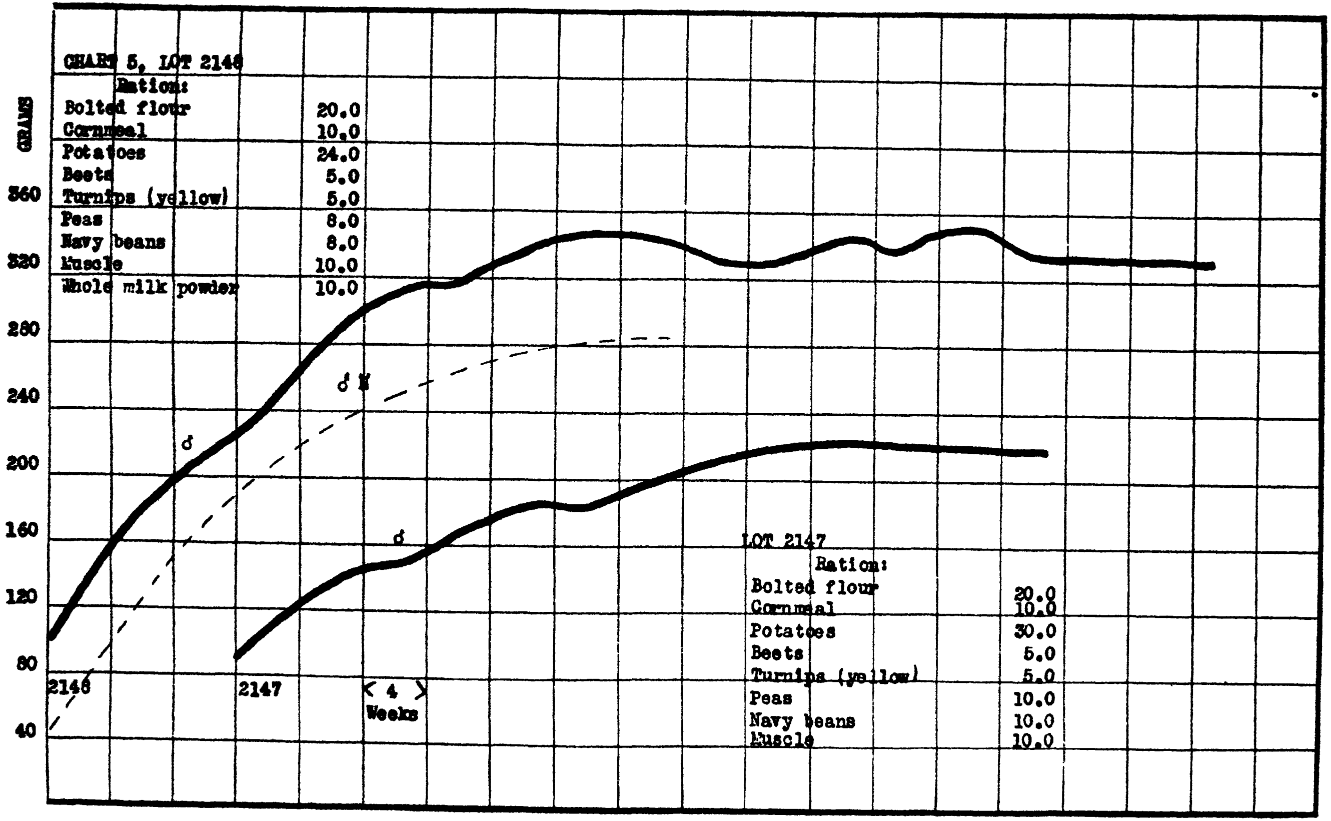
CHART 5, LOT 2146
Ration:
Bolted flour 20.0
Cornmeal 10.0
Potatoes 24.0
Beets 5.0
Turnips (yellow) 5.0
Peas 8.0
Navy beans 8.0
Muscle 10.0
Whole milk powder 10.0
GRAMS
360
320
280
240
200
160
120
80
40
♂
♂ M
♂
2146
2147
< 4 >
Weeks
LOT 2147
Ration:
Bolted flour 20.0
Cornmeal 10.0
Potatoes 30.0
Beets 5.0
Turnips (yellow) 5.0
Peas 10.0
Navy beans 10.0
Muscle 10.0

STUDIES IN THE VITAMINE CONTENT.

II. THE YEAST TEST AS A MEASURE OF VITAMINE B.

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(Received for publication, March 30, 1921.)

Since Williams (1) first suggested the identity of Wildier's (2) "bios" with water-soluble B vitamine and proposed as a test for the vitamine the measurement of yeast stimulation, the procedure has undergone considerable investigation and criticism. As a result we now have a choice of several methods of applying the test which may be classified as follows: Measurement of yeast stimulation (*a*) by counting the cells; (*b*) by weighing the cells produced (1, 3); (*c*) by determination of the CO_2 produced (4); and (*d*) by determination of the volume of cells produced (5, 6). Of the methods presented to date the authors have found that the method devised by Funk and Dubin (5) combines quantitative accuracy with the simplest technique. In the work reported here we have applied this method.

In the field of criticism various types of questions and doubts have been advanced as to the specificity of the test and its adaptability as a quantitative instrument for vitamine measurement. The principal objections to its use may be reviewed briefly. Souza and McCollum (7) call attention to the sensitivity of yeast cells to many types of stimulation and advance data to illustrate this view as evidence against the accuracy of the test for evaluating vitamine content. More recently MacDonald and McCollum (8) have shown by a series of transplants that yeast will grow in a culture medium practically devoid of all but a trace of "bios" and suggest that this result is explicable on one of two hypotheses; *viz.*, that either yeasts do not require "bios" for growth or that they can synthesize enough to meet their needs. Either explanation would make the test inaccurate for determining total vitamine in an extract.

Emmett and Luros (9) and Emmett and Stockholm (10) deny that the "bios" is vitamine B and present evidence to show that the stimulative factor possesses neither antineuritic power nor the power to induce growth in white rats. They grant the presence of a stimulative factor in extracts of vitamine-rich materials but imply that it is either a new vitamine or a factor of another sort.

A series of articles has recently appeared reporting work by three authors, Fulmer, Nelson, and Sherwood (11). In these articles they point out that the stimulative effects of alfalfa and wheat embryo extracts upon yeast growth are not directly proportional to the weights of the materials extracted. Funk had already called attention to this fact in his article describing his centrifugation method. Second, they present evidence tending to show that the stimulative effect of an extract or culture medium is greatly altered by varying the concentrations and kinds of mineral salts therein. In this connection they propose a formula for a culture medium (Medium F) which they believe will produce maximum stimulation to yeast growth and which will not be improved or altered by the addition of extracts of organic substances unless the latter disturb the optimum salt concentrations of the medium. In brief they imply that "bios" is a matter of salt concentration. Third, they advance the claim that the stimulative effect of alfalfa extracts upon growth of yeast cells cannot be changed by the treatment of the extract with alkali and this fact indicates that the stimulative factor cannot be vitamine B.

Should all these criticisms prove valid it would seem idle to proceed further with the development of the test. In reviewing these papers and our previous work we are not yet convinced that the time has come to reject the test as worthless. In the present paper we wish to present our accumulated data of the past 6 months which have been devoted to somewhat detailed investigations of behavior of the test under varying conditions.

Since the presentation of the Funk-Dubin method (6) at New Haven in May, 1920, we have adopted that technique. For various reasons we have modified the reported method somewhat, but in the main it is essentially as described by the authors. Since, however, all the results of this article are based on the use of this test, it may be worth while to briefly describe our procedure.

Method.

To a basal diet of 9 cc. of sterile culture medium (Nägeli solution, Medium F, etc.) in a sterile test-tube is added 1 cc. of the sterilized extract of the material to be examined. A pure culture of Fleischmann yeast is maintained on an agar slant and 24 hours before the test is to be made, a transplant is made to a fresh agar slant. (Funk and Dubin hold that brewer's yeast is better adapted to the purposes of the test since it is always bottom growing and packs more readily on centrifugating.) One standardized platinum loopful of the 24 hour yeast is used to inoculate the contents of each tube, the tube is then stoppered with cotton and incubated. In the experiments that follow, unless otherwise stated, the culture medium used by us has been the Nägeli solution. Our incubators are set for 31°C. and are so maintained that their maximum temperature variation lies between 30 and 35°C. For various reasons we have selected 72 hours as our period of incubation. At the end of the incubation period the yeasts are killed by plunging the tubes into water heated to 80°C. and the tubes are maintained at this temperature for 15 minutes. Unless the yeasts are killed they will not pack well in the subsequent centrifugation.

The contents of the tube are next transferred to a Hopkins' vaccine centrifuge tube which has a capillary tip graduated from 0.00 to 0.05 cc. with five markings. With a magnifier and a scale it is possible to read accurately to tenths of the divisions etched on the tip; *i. e.*, to thousandths of a cc. The tube and its contents are then centrifugated 20 minutes at a speed of about 2,500 revolutions per minute.

In the preparation of our extracts various methods were employed depending upon the nature of the source. We have found that boiling water will in 3 hours extract all the yeast stimulative substance from a small amount of dried material unless considerable starch is present. In that case we have resorted to extraction with 95 per cent alcohol, filtered, evaporated to dryness on a steam bath, taken up the residue with water, filtered again, and diluted or concentrated the filtrate as desired. If much fat is present a preliminary extraction with alcohol-free ether will prepare the material for water or alcohol extraction. We have used each and all of these methods as the circumstances warranted. When extracting food materials it is customary to dry them to constant weight at 60°C. in an air bath. The filtered watery extracts used in the tests are determined colorimetrically for pH. In practice, if the hydrogen ion concentration falls between a pH of 6 and one of 7 no further treatment is applied to the extract. If the concentration is over 7 or under 6 we have neutralized with HCl to litmus and restored to the original volume by dilution or evaporation. Excess of alkali not only retards yeast growth but also tends to precipitate the calcium phosphates from the culture medium. Once prepared, the extracts are placed in cotton stoppered Erlenmeyer flasks and sterilized in an Arnold steam sterilizer. Two 30 minute treatments with a 24 hour interval are usually sufficient to secure sterility and once sterilized the flasks and contents can be kept indefinitely.

I.

Our first experiments aimed to apply the above technique to a series of materials whose relative vitamine B content had been already established by rat-feeding tests. We, therefore, selected for this purpose the materials described by Osborne and Mendel (12) in a previous article in this Journal. Our tests with these sources fall into three series. In our first series we obtained fresh materials of the kind described by Osborne and Mendel, dried them at 60°, and made our extracts by boiling the dried materials with water. These preparations are those listed on the chart as "Dr. Heft's preparations." To make sure that our extraction was complete we reextracted the residues until tests showed that the stimulative factors were reduced to the limits of the controls. Since water was used in the experiments it does not follow that we obtained all the vitamine that a rat gets out in digestion, but with that reservation, we feel sure that our tested extracts contained all of the vitamine that was extractable by water. The final extracts were combined and concentrated to a volume such that 50 cc. contained the extractable

TABLE I.

A comparison of the vitamine values of a series of materials as determined by Osborne and Mendel (12) in a rat-feeding experiment with the values given by the yeast test applied to water extracts of similar materials (Dr. Heft's preparations).

The Osborne and Mendel results.

Materials used	Gain of weight of rats fed 1 gm daily				Total	Average		Gain of weight of rats fed 0.5 gm daily			Total	Average	
	1	2	3	4				1	2	3			
Alfalfa . . .	200	179	146		525	175	1	144	121	106	371	124	1
Clover . . .	197	177	150		524	174	2	112	102	99	313	99	2
Tomato . .	122	120	118	115	475	119	3	88	70		158	79	3
Spinach . .	119	101	81	75	376	94	4	87	61	32	180	60	4
Cabbage...	99	91	58		248	82	5	52	49	30	131	43	5
Turnip......	96	68	66		230	77	6	46	30	20	96	32	7
Carrot	78	78	66	46	268	67	7	49	38	36	123	41	6
Timothy . .	42	40	10		92	31	8	22	5	−2	25	8	8
Beet	6	−9	−3		−6	−2	9	No data on 0.5 gm.					

The yeast test results. 1 cc. of water extract contained the extractable "bios" of 0.02 gm. dried material 1 cc. of extract and 9 cc. of Nageli solution were used in each determination.

Materials in Osborne and Mendel order of value.	Individual determinations in thousandths of a cc. of yeast cells										Total	Average	Rating
	1	2	3	4	5	6	7	8	9	10			
Alfalfa.	60	45	33	32	32	31	31	31	30	29	354	35	5
Clover.. ...	58	48	46	45	43	40	40	40	38	38	436	44	3
Tomato	55	55	49	48	42	42	39	39	39		408	45	2
Spinach......	82	81	76	70	70	69	66	63			577	72	1
Cabbage......	46	41	33	32	26	26	25	23			252	31	7
Turnip.	38	38	37	35	34	34	33	28	28		305	34	6
Carrot.......	51	50	42	42	42	41	41	40	40		389	43	4
Timothy....	42	40	33	31	27	25	25	23	22	22	290	29	8
Beet.........	29	26	26	24	24	23	22	22	18		214	24	9
Control......	23	15	15	15	12	8	8	23	2	1	122	12	

material from 1 gm. of dried material. Table I shows the results obtained with the materials of the first series and Chart I is a graphic presentation of the same.

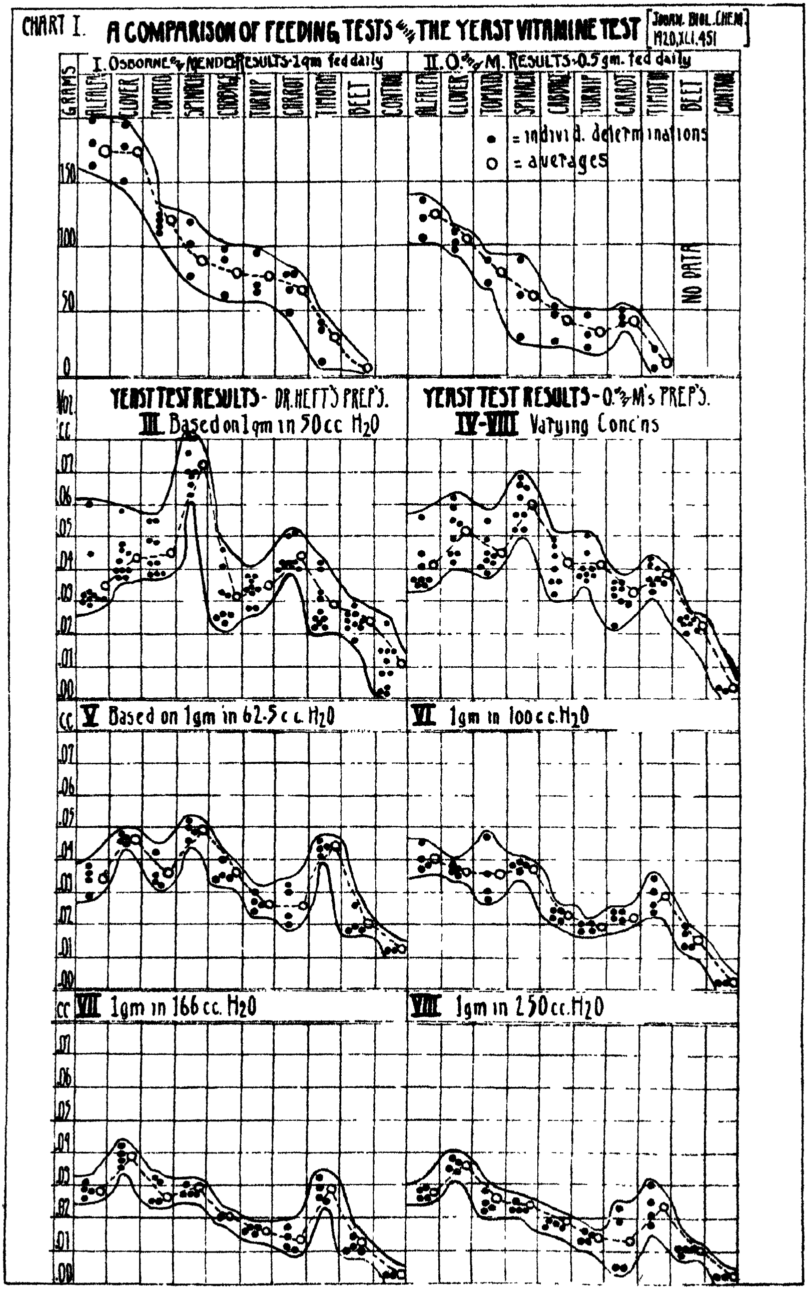


CHART I. A COMPARISON OF FEEDING TESTS with THE YEAST VITAMINE TEST
[Journ. Biol. Chem. 1920, XLI, 451]
I. Osborne and Mendel Results - 1 gm fed daily
II. O. and M. Results - 0.5 gm. fed daily
GRAMS
ALFALFA
CLOVER
TOMATO
SPINACH
CABBAGE
TURNIP
CARROT
TIMOTHY
BEET
CONTROL
150
100
50
0
● = individ. determinations
○ = averages
NO DATA
YEAST TEST RESULTS - DR. HEFT'S PREP'S.
III. Based on 1 gm in 50 cc H2O
YEAST TEST RESULTS - O. and M's PREP'S.
IV-VIII Varying Conc'ns
Vol. cc
.07
.06
.05
.04
.03
.02
.01
.00
cc
V Based on 1 gm in 62.5 c.c. H2O
VI 1 gm in 100 c.c. H2O
VII 1 gm in 166 cc. H2O
VIII 1 gm in 250 cc. H2O

While we may justifiably draw from these results an approximate agreement with the Osborne-Mendel conclusions the agreements are not exact and in the case of spinach there is a very marked discrepancy. We felt at the time that our variations might quite possibly be due to variability in the vitamine content of our materials as compared with the Osborne and Mendel

TABLE II.

Results of the yeast test applied to varying concentrations of water extracts of the actual materials used in the Osborne and Mendel experiments.*

A. 1 cc. of extract tested carried the extractable "bios" of 0.02 gm. dried material. 1 cc. extract plus 9 cc. Nägeli solution used in each test.

Materials in Osborne and Mendel order of value.	Readings in thousandths of a cc.								Total	Average	Order of value.
	1	2	3	4	5	6	7	8			
Alfalfa..........	56	45	41	37	37	37	36	36	325	41	4
Clover	62	59	55	54	49	45	45	42	411	51	2
Tomato	55	49	45	44	43	42	41	39	358	45	3
Spinach	68	66	65	62	57	57	52	52	479	59	1
Cabbage	49	47	44	40	36	36	32		284	40	6
Turnip..	50	45	40	40	39	38	38	37	327	41	5
Carrot	36	36	34	34	32	30	29	22	253	31	8
Timothy	43	41	40	37	37	36	35	33	302	37	7
Beet	25	24	24	23	23	21	20		160	22	9
Control..	2	3							5	2 5	10

	B. Concentration: 1 cc. carries "bios" of 0.016 gm. dried extract.							C. Concentration: 1 cc. carries 0.01 gm.						
Materials	1	2	3	4	Total	Average	Order.	1	2	3	4	Total	Average	Order
Alfalfa...........	38	36	34	29	137	34	6	37	38	40	45	160	40	1
Clover...........	45	46	47	48	186	46	2	35	36	37	38	146	36	3
Tomato..........	32	33	35	42	142	35	5	28	30	35	47	140	35	4
Spinach..........	46	49	50	52	197	49	1	36	38	38	39	151	37	2
Cabbage.........	34	34	35	40	143	36	4	21	22	24	24	91	22	6
Turnip...........	24	26	27	30	107	27	7	18	18	20	20	76	19	8
Carrot............	20	22	30	32	104	26	8	21	22	24	24	91	22	7
Timothy..........	41	42	43	46	172	43	3	23	26	30	34	113	28	5
Beet.............	18	18	19	26	81	20	9	13	13	17	19	62	15	9
Control..........	2	2			4	2	10	2	2			4	2	10

TABLE II—*Concluded.*

	D. Concentration: 1 cc. carries "bios" of 0.006 gm. dried material.							E. Concentration: 1 cc. carries 0.002 gm.						
Materials.	1	2	3	4	Total.	Average	Order.	1	2	3	4	Total.	Average	Order.
Alfalfa	26	28	29	31	114	28	2	26	26	28	29	109	27	2
Clover	36	38	40	42	156	39	1	34	35	37	38	144	36	1
Tomato	25	25	31	32	113	28	3	22	23	24	28	97	24	3
Spinach	27	27	27	30	111	27	5	22	22	22	25	91	22	5
Cabbage	20				20	20	6	17	17	18	19	71	18	6
Turnip	15	16	17	17	65	16	7	13	13	15	16	57	14	7
Carrot	10	11	14	17	52	13	8	5	5	19	23	52	13	8
Timothy	25	26	29	32	112	28	4	18	21	25	30	94	23	4
Beet	10	10	11	14	45	11	9	9	10	10	10	39	9	9
Control	2	2			4	2	10	2	2			4	2	10

* For graphic presentation see Chart I.

sources. To check this we sought a test upon the actual materials used in the Osborne and Mendel experiments and these materials were kindly furnished us for the purpose by Professor Mendel. With the actual materials at hand we repeated our tests and in this set of experiments we varied our procedure by testing various concentrations of each extract. Table II gives the actual results and these are also reproduced graphically in Chart I.

The most significant features of these results are the extreme variability in individual determinations in the higher concentrations, lessened variability, and closer approximation to the feeding test results when diluted extracts are used. The behavior of the diluted concentrations is we think more than a coincidence. Funk (5) and others (11) have shown that the curve of stimulation under varying concentrations approximates the logarithmic curve and that to get sharp contrasts between two extracts it is necessary to test dilutions which will fall on the steep part of the curve. This point is emphasized in the next series of experiments.

II.

The results with the varying concentrations suggested that we proceed next to the establishment of the curve of reaction of a single material. To this end we first selected dried alfalfa meal as our material and, keeping all factors constant except concentration, proceeded to determine the stimulative activity in each concentration. To obtain our extract, 400 gm. of dried alfalfa meal were repeatedly extracted with boiling distilled water, the filtered extracts combined, refiltered, and concentrated to a degree that 1,000 cc. contained the extractable material from 400 gm. In working with this extract we were at first bothered by the tendency to sedimentation on standing or centrifugating. To avoid this complication we employed a procedure described by Osborne and Wakeman for removing protein from spinach extracts. This consisted in diluting the water extract with 95 per cent alcohol to 40 per cent of the volume and filtering off the precipitated protein complex. This procedure is attended with variable results. Sometimes the precipitate carries down with it all the stimulative substance in the extract. At other times very little loss results. In the extract which we used tests showed that the loss was less than 0.002 cc. expressed in terms of yeast tests made before and after treatment. The alcohol treatment naturally diluted our extract and to remove the alcohol and restore the desired concentration the filtrate was concentrated on the water bath to the original 1,000 cc. The tests reported below were made on this material and variations in concentration are expressed as fractions of the maximum concentration. Table III and Chart II show six series of tests. In Series A, B, D, and E each determination was made by combining 1 cc. of the alfalfa extract with 9 cc. of Nägeli solution. In Series C, Fulmer, Nelson, and Sherwood's Medium F was substituted for the Nägeli solution. Series F was obtained with a filtered autolysate of Fleischmann yeast cakes. One pound of the moist cakes was mixed with water and allowed to autolyze. The mixture was then filtered and concentrated to such a volume that the highest concentration used represents the extractable material from 3 gm. of Fleischmann yeast cake in each cc. of extract.

The general shape of the curves of Series A and B is the same, the only difference being in the height of Series B and in the lower control value with this extract, possibly due to repeated heating in the 2 months that elapsed between the two tests. Both show a steep ascent to an optimum with little variability to the left of the optimum. On the right of the optimum there is not only much wider variation in the test results but a marked decline. These results confirm those of Fulmer, Nelson, and Sherwood (11). These peculiarities show the futility of attempting to compare the value of two extracts unless the curves of the two are known and the impossibility of depending upon comparisons based on weights

TABLE III.

The yeast growth curve determined by varying the concentrations of a given extract. Time of incubation 72 hours in each determination. The results are given in thousandths of a cc. of yeast cells.

Series A. Alfalfa extract plus Nägeli solution. Maximum concentration represents the extractable material from 0.4 gm. dry alfalfa meal in 1 cc. of water.

Concentration .	1	0.9	0.8	0.7	0.6	0.5	0.45	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.05	Control
	22	23	27	30	24	24	29	34	36	35	36	29	30	21	20	6
	23	24	28	30	25	28	31	35	38	37	36	30	34	21	22	7
	24	28	31	33	28	28	33	35	39	37	36	32	34	27	25	6
	26	29	33	35	35	29	35	37	39	37		32		27	30	7
						29		37		39		33		27		6
						31		38		40		34		31		5
						35		38		40		34		31		
						36				41		35		32		
Total........	95	104	119	128	112	240	128	254	152	306	108	259	98	220	97	37
Average.......	24	26	29	32	28	30	32	36	38	38	36	32	32	27	24	6

Series B. Made 2 months later but with the same extract of alfalfa used in the Series A tests.

Concentration	0.95	0.75	0.65	0.55	0.475	0.425	0.375	0.325	0.275	0.225	0.175	0.125	0.075	0.045	0.035	0.025	0.015	Control.
	17	24	25	23	28	28	30	29	28	29	23	25	21	18	16	13	14	2
	20	26	25	25	29	29	33	33	29	29	27	26	21	18	16	15	15	2
	20	27	26	30	34	34	33	33	29	30	29	26	22	19	17	15	17	
	21	28	26	30	35	35	34	34	32	30	30	27	22	19	18	15	17	
	25	28	27	31	36	36	34	35	35	32	33	29	24	19	19	16	18	
Total.......	103	133	129	139	162	162	164	164	153	150	142	133	110	93	86	74	81	4
Average.....	20	27	26	28	32	32	33	33	31	30	28	27	22	19	17	15	16	2

TABLE III—*Continued*

Series C. Made with the same extract of alfalfa as Series A and B but with Fulmer, Nelson, and Sherwood's Medium F in place of Nageli solution.

Concentration	0 95	0 75	0 65	0 55	0 475	0 425	0 375	0 325	0 275	0 225	0 175	0 125	0 075	0 045	0 035	0 025	0 015	Control.
	30	37	32	34	30	32	28	28	26	27	26	23	20	18	18	17	18	13
	37	38	32	35	30	33	29	29	30	28	26	25	22	18	19	18	19	14
	42	42	42	35	31	34	29	32	30	28	27	25	23	20	20	19	20	15
	43	55	44	46	34	34	33	33	30	30	29	26	23	20	20	19	20	16
	62	55	45	47	36	35	35	34	33	30	29	26	23	22	21	20	21	17
Total . .	214	227	195	197	161	168	154	156	149	143	137	125	111	98	98	93	98	75
Average.. .	43	45	39	39	32	33	31	31	29	28	27	25	22	19	19	18	19	15

Series D and E were made with alfalfa extract and Nageli solution at the same time as Series B in order to determine the curvature of the steep part of the alfalfa curve. A concentration of 0 1, therefore, represents the extractable material from 0.04 gm. alfalfa in 1 cc. of water.

Series D.

Concentration .	0 1	0 09	0 08	0 07	0 06	0 05	0 04	0 03	0 02	0 01	Control
	25	20	22	20	18	16	15	14	13	10	2
	25	22	22	21	18	16	16	15	14	10	2
	25	23	23	21	19	17	16	15	14	10	
	25	25	23	22	20	19	16	16	14	10	
	25	25	24	22	20	19	16	16	14	11	
Total.............	125	115	114	106	95	87	79	76	69	51	4
Average.............	25	23	23	21	19	17	16	15	14	10	2

Series E.

Concentration	0.01	0.009	0 008	0 007	0 006	0 005	0 004	0 003	0 002	0 001	Control
	9	9	8	8	7	7	5	4	3	2	2
	9	9	8	8	7	7	6	5	4	3	2
	9	9	9	8	7	7	7	6	5	4	
	10	9	9	8	7	7	6	5	5	3	
	10	10	9	8	7	7	6	5	5	3	
Total...............	47	46	43	40	35	35	30	25	22	15	4
Average.............	9	9	8	8	7	7	6	5	4	3	2

TABLE III—*Concluded*

Series F. Made with yeast autolysate in place of alfalfa extract. In the maximum concentration 1 cc. of autolysate contains the extractable substance from 3 gm. of Fleischmann yeast cake. In this series the autolysate was combined with Nageli solution.

Concentration	1	0.64	0.56	0.48	0.4	0.32	0.24	0.2	0.16	0.12	0.1	0.09	0.08	0.07	0.05	0.04	0.03	0.02	0.01	Control
	68	44	62	68	35	63	60	65	52	65	69	55	27	32	47	45	41	28	23	2
	68	70	63	70	36	65	68	80	60	69	70	69	36		48	47	42	39	30	2
	70			70	38	68	68		67	80			46							2
	70				52	70	70		67	80			46							2
	70				70	75	70		69				50							
							70		70				56							
Total..	346	114	125	208	231	341	406	145	385	294	139	124	261	32	95	92	83	67	53	8
Average..	69	57	62	69	46	68	67	72	64	73	69	62	43	32	47	46	41	33	26	2

of extracted materials as already pointed out by Funk and Dubin (5) and Fulmer, Nelson, and Sherwood (11). The shape of the curve also established the following data concerning the behavior of alfalfa extract. First, that no matter how concentrated the extract, it was impossible to develop a stimulative effect greater than the production of about 0.04 cc. of yeast cells in 72 hours for each cc. of extract used; second, that increasing the concentrations beyond the optimum lowers the production of yeast. The question naturally arises as to whether these features are common to all extracts; whether the decline to the right of the optimum is due to hydrogen ion effect; and whether time of incubation is a factor? The yeast autolysate curve shows that while its shape is essentially the same as that of alfalfa in showing steep rise, optimum concentration, and decline in higher concentration, its power to produce growth of yeast cells exceeds the optimum power of alfalfa extract. This fact alone would be sufficient to show that in making the yeast test we are concerned with other factors than the presence of vitamine B and its concentration. In Table IV are given the results obtained by applying the test to a variety of materials.

CHART II. EFFECT OF VARIATION IN CONCENTRATION ON THE TEST

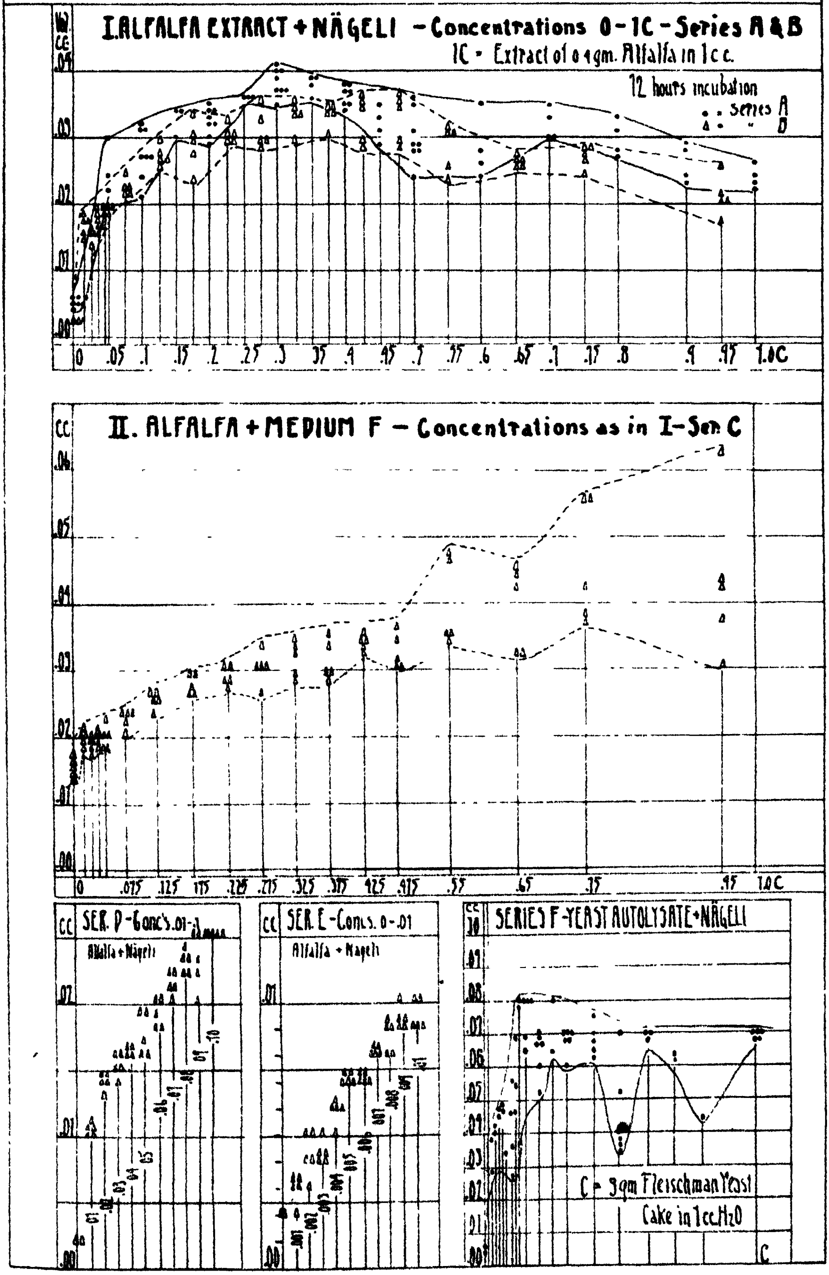


TABLE IV.

Results of applying the yeast test to varying concentrations of extracts of the materials named.

Test results with a dilute water solution of Funk's 1913 crystalline complex (vitamine). 0.0079 gm. was dissolved in 75 cc. of water. This solution strength is marked 1 in the series below and further dilutions were made for test purposes as indicated. The results are given in thousandths of a cc.

Concentration.	1	0.8	0.5	0.4	0.3	0.2	0.1	0.08	0.05	0.03	Control.
	7	7	6	5	4	4	4	4	4	4	2
	7	7	6	6	6	4	5	4	4	4	2
	7	7	7	6	6	5	5	4	4	4	
	7	7	7	6	6	5	5	4	4	4	
	8	8	7	6	6	5	5	4	5	4	
Average...........	7	7	6	5	5	4	4	4	4	4	2

A comparison of extracts made from gland material extracted first with neutral alcohol and the residue reextracted with acidified alcohol.

	1. Neutral alcohol extracts original glands					Gland.	2. Acid alcohol extracts of the residues from 1.				
Concentration....	C	0.8C	0.5C	0.2C	Control		C	0.8C	0.5C	0.2C	Control.
	20	22	23	18	5	Sheep pancreas. C equals 2 gm. fresh gland in a cc.	13	13	13	11	3
	29	23	24	20	6		13	15	14	11	2
Average.......	24	22	23	19	5		13	14	13	11	2
	15	13	9	7	5	Human pancreas.	10	9	8	5	3
	15	14	13	7	6		10	10	8	6	2
Average.......	15	13	11	7	5		10	9	8	5	2
	9	8	6	4	5	Human liver.	8	7	5	4	3
	10	8	7	4	6		8	7	7	4	2
Average.......	9	8	6	4	5		8	7	6	4	2

The only new point brought out by these tests relates to the gland extracts. Swoboda (13) in a recent article in this Journal advanced the view that Williams' results with pancreas were due to his use of acid in making his extract, since with the method employed he was unable to get stimulative effects on yeast growth with pancreatic extract. He formulated a hypothesis of vitamine existing as a vitaminogen. The tests in Table IV were designed primarily to test this view. The results fail to confirm either of his contentions. We get stimulation well above the control with a neutral alcohol extract of pancreas and the residues on reextraction with acidified alcohol yield extracts of lower potency. If the vitaminogen view were correct the latter should exceed the first in power. The concentrations used were not sufficiently great to add much data to the question of curve shape, or location of optima. A study, however, of the tests with the Osborne and Mendel materials enables us to conclude that the optima vary with the material extracted.

The decline of growth in our concentrations of alfalfa extract higher than the optimum could not be due to the pH, for its values (determined colorimetrically) in these inhibitory concentrations differed too little from that of the optimum concentration to function as an inhibitory factor in this experiment.

To answer the query as to the effect of time of incubation on our results we tested out the rate of growth of yeast cells in two concentrations of the alfalfa extract as shown in Table V and Chart III. The concentrations selected were the one found optimum in the Series B tests and the full concentration of the extract. The results show that after 24 hours and up to a period of 19 days the time factor does not change the result. The concentration found optimum in the earlier tests retains its superior stimulative potency regardless of the time of incubation.

These results do not answer all the questions raised by the alfalfa curve. They do, however, make it clear that if vitamine B is one of the factors concerned in the combined "bios" effect of an extract it is only one of several factors concerned and that the use of the yeast test for quantitative measurement of vitamine content must await the development of a medium which shall be optimum for all factors except vitamine, exactly the situation that had to be met in the development of basal diets for rat-feeding experiments.

III.

The assumption that it is possible to develop a basal diet for yeast cells, optimum in all except vitamine B implies that at least one of the stimulative factors is vitamine B. If Fulmer, Nelson, and Sherwood's contentions (11) are true even this factor is eliminated and further attention to development of the test is useless. Their contentions rest upon two different pieces of evidence; *viz.*, the claim that by proper selection of a medium

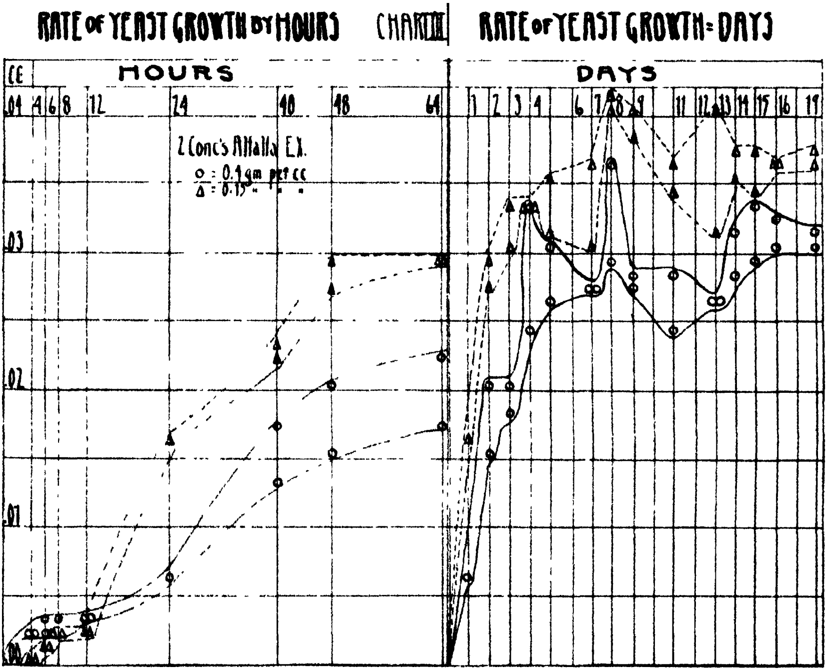
TABLE V.

The effect of the period of incubation on the rate of growth of yeast cells in the optimum concentration of alfalfa extract as contrasted with the higher concentration of the extract.*

Period of incubation	Concentration I 1 cc contains the extract of 0.4 gm of alfalfa			Concentration II (optimum) 1 cc contains the extract of 0.15 gm of alfalfa.		
	Test.	Test	Average	Test	Test	Average
hrs						
4	2	2	2	0	0	0
6	2	3	2.5	1	1	1
8	2	3	2.5	2	2	2
12	3	3	3	2	2	2
24	6		6	16		16
40	13	17	15	22	23	22.5
48	15	20	17.5	27	29	28
64	17	22	19.5	29	29	29
days						
1	6		6	16		16
2	15	20	17.5	27	29	28
3	18	20	19	30	33	31.5
4	29	33	31	38	38	38
5	26	30	28	32	35	33.5
7	27	27	27	30	36	33
8	28	36	32	40	41	40.5
9	27	26	26.5	38	40	39
11	24	28	26	34	36	35
13	26	26	25	31	40	34.5
14	28	31	27.5	35	37	36
15	29	33	31	34	37	35
16	30	32	31	36	36	36
19	30	31	30.5	36	37	36.5

* For graphic presentation see Chart III.

of known constituents it is possible to obtain a medium that will produce a growth of yeast cells which is not improved by the addition of organic extracts, and second that the stimulative power of extracts on yeast growth is not affected by alkali treatment of these extracts, vitamine B being known to be extremely sensitive to the destructive effect of alkali, especially when combined with heat.



To settle these points we felt that a fair test would be to substitute for the Nägeli solution the Medium F of Fulmer, Nelson, and Sherwood and see if our tests would still yield increased growth phenomena when this medium was supplemented with alfalfa extract. The differences in the two media are shown below.

Nägeli solution.	Medium F.
100 cc. distilled water.	100 cc. distilled water.
1 gm. ammonium nitrate.	0.188 gm. ammonium chloride.
0.05 " calcium phosphate.	0.10 " calcium chloride.
0.5 " K_2HPO_4.	0.10 " K_2HPO_4.
0.25 " magnesium sulfate.	0.04 " precipitated calcium carbonate.
10.0 " dextrose.	0.6 " dextrin.
	10.0 " sucrose.

In using the F medium it is obvious that the calcium carbonate will sediment with the yeast cells unless removed before centrifugating. We accomplished this by adding dilute HCl, drop by drop, to the tube contents just before centrifugating. The results of this test with varying concentrations of alfalfa extract added, are shown in Series C of Table II and in Chart II. They show that while, as evidenced by the control figures, Medium F is superior to Nägeli solution as a basal medium it is still capable of stimulation by every concentration of the alfalfa extract used and that while the optimum growth obtained is greater than that obtained with Nägeli solution the shape of the curve is essentially the same as with Nägeli solution. These results seem at least to make questionable Fulmer, Nelson, and Sherwood's first contention.

To test their contention regarding the effect of alkali we selected both alfalfa extract and yeast autolysate for experimental materials. Our alkali-treated extracts were obtained in two ways. A portion of the extract was measured out and make up to 10 per cent strength with solid NaOH. This mixture was then autoclaved at 15 pounds above atmospheric pressure for 3 hours. After autoclave treatment the extract was neutralized with HCl to litmus and evaporated to its original concentration. Except for the action of the alkali on the components of the extract the sole change made in composition was an increase in NaCl. 1 cc. portions of the treated extract, diluted to provide varying concentrations, were then combined with 9 cc. of Nägeli solution in one experiment and with Medium F in another. Previous experiments had provided us with the control data (see Table II and Chart II). Table VI and Chart IV give the results.

TABLE VI.

The effect of alkali treatment upon the stimulative powers of alfalfa and yeast extracts.*

Studies of alfalfa extracts. (C equals the extractable material from 0.4 gm. of dried alfalfa in 1 cc. of water.)

	Concentrations used.	Tests with alkalied extracts	Average	Average untreated extract
Nägeli solution used.	0.95C	20 26 28	24	20
	0.75C	22 27 27	25	27 See Table III.
	0.55C	27 27 27	27	28
	0.475C	26 27 28	27	32
	0.375C	25 27 31	28	33
	0.275C	23 25 25	24	31
	0.175C	20 25 25	23	28
	0.075C	16 16 17	16	22
	0.035C	14 14 17	15	17
	0.015C	10 10 11	10	16
	Control.	2 2 2	2	2
Medium F used.	0.95	22 23 24	23	43
	0.75	21 22 28	24	45
	0.55	18 22 26	23	39
	0.475	20 24 26	23	32 See Table III.
	0 375	18 20 21	19	31
	0.275	15 16 18	16	29
	0.175	11 12 13	12	27
	0.075	10 12 12	11	22
	0.035	11 11 12	11	19
	0.015	9 10 10	10	19
	Control.	4 4 4	4	15

Studies of yeast autolysate. (C equals 0.75 gm. in 1 cc.)

Concentrations used.	Alkali-treated with Nägeli	Average	Alkali-treated with Medium F	Average
0.95C	23 25 33	27	22 23 23	22
0.05C	22 24 25	24	17 17 18	17
0.475C	20 23 23	22	15 15 16	15
0.375C	20 25 26	24	13 15 16	15
0.275C	18 18 20	18	12 12 13	12
0.175C	17 17 18	17	11 11 10	11
0.075C	13 13 13	13	10 10 11	10
0.035C	8 9 9	9	8 9 9	9
0.015C	2 2 2	2	2 7 7	5
Control.	2 2	2	3 3 3	3

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* For comparison with untreated yeast autolysate see Chart IV.

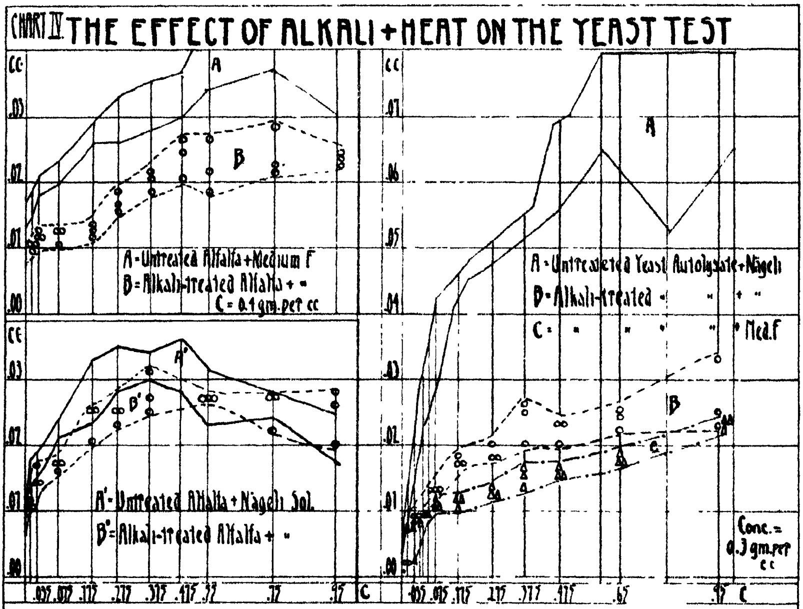
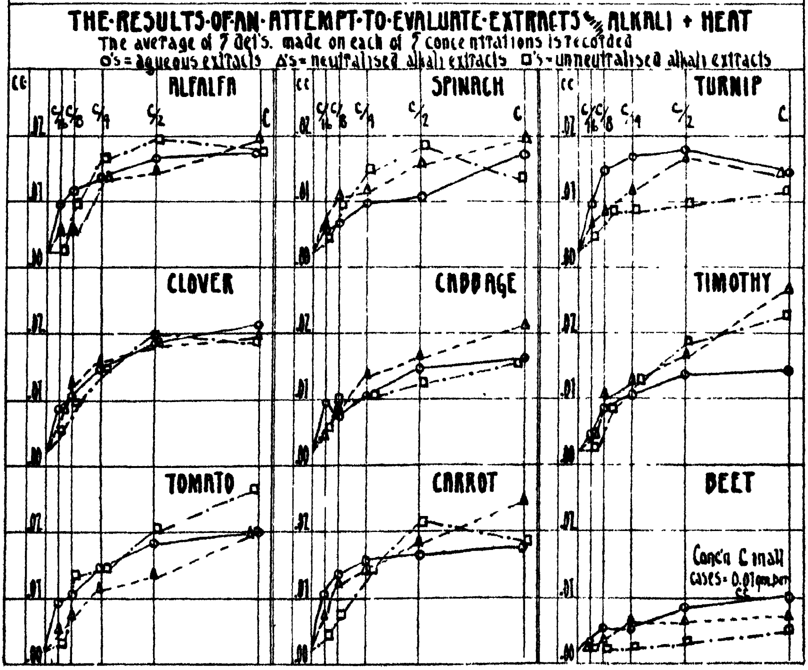


CHART IV THE EFFECT OF ALKALI + HEAT ON THE YEAST TEST
cc
.03
.02
.01
.00
A
B
A - Untreated Alfalfa + Medium F
B = Alkali-treated Alfalfa + "
C = 0.1 gm. per cc
A'
B'
A' - Untreated Alfalfa + Nägeli Sol.
B' = Alkali-treated Alfalfa + "
.035 .075 .115 .215 .315 .415 .55 .75 .95 C
.07
.06
.05
.04
A - Untreated Yeast Autolysate + Nägeli
B - Alkali-treated " " + "
C = " " " " + Med. F
Conc. = 0.3 gm. per cc
.005 .015 .115 .215 .315 .415 .65 .95 C


THE·RESULTS·OF·AN·ATTEMPT·TO·EVALUATE·EXTRACTS % ALKALI + HEAT
The average of 7 det's. made on each of 5 concentrations is recorded
O's = aqueous extracts Δ's = neutralised alkali extracts □'s = unneutralised alkali extracts
ALFALFA
SPINACH
TURNIP
CLOVER
CABBAGE
TIMOTHY
TOMATO
CARROT
BEET
cc
.02
.01
.00
C/16 C/8 C/4 C/2 C
Conc'n C in all cases = 0.07 gm. per cc

When these results are examined carefully the conclusions are not so definite as the figures might at first indicate. They seem to show destruction by the alkali of some of the power but the extent of the destruction is not nearly so great as would be expected if vitamine B formed a large part of the stimulative factors. It is also easy to see why Fulmer, Nelson, and Sherwood reached their conclusion as to practically no destruction if they tested only the higher concentrations, for in the higher concentrations the effect is much less marked, especially in the experiments with Nägeli solution as the basal medium. These facts show up more distinctly in the charted results. We therefore resolved to make another test. If vitamine B is one of the factors and if it is destroyed by alkali the extracts of the Osborne and Mendel series ought to show a marked reduction in stimulative power after alkali treatment. To determine this we made a new set of extracts of those materials. These sets consisted of three series obtained as follows: Series A represents aqueous extracts of 1 gm. of dried material made up with water so that 1 cc. carried the "bios" of 0.01 gm. of dried material. Series B consists of a similar set obtained by extracting 1 gm. of the dried material with 0.1 N NaOH, filtering, neutralizing with HCl to litmus, and diluting until 1 cc. carried the "bios" of 0.01 gm. of dried material. In both these extractions the material was boiled continuously for 3 hours in the extractant. The third series was obtained by taking 50 cc. portions of Series B, adding 5 cc. of 0.1 N NaOH to each portion, heating at 100°C. for 30 minutes, and then testing without neutralizing. We assumed that the order of test effects would be in all cases highest with Series A, Series B coming next, and Series C third. The actual readings are given in Table VII and graphically in Chart IV.

These results fail to confirm the conclusions from Table VI and seem to give excellent confirmation of Fulmer, Nelson, and Sherwood's view that the stimulative factor is not appreciably affected by alkali treatment. These two sets of experiments, therefore, leave that question unsettled.

From these results and from the previous ones it becomes increasingly evident that the growth of the yeast cells, as determined by any one of the methods devised to date, is a resultant of so many different factors, mutually interacting, that to interpret these growths as a quantitative measure of vitamine content is unjustified. All the evidence except that based on alkali treatment seems to argue in favor of vitamine B as at least one of the growth stimulants while the alkali evidence is contradictory and incomplete. There seems, therefore, only one alternative; namely, to attempt to separate and identify the factors concerned in the growth stimulation and if possible devise a medium that

TABLE VII.

Effect of alkali treatment on the Osborne-Mendel preparations. Only the averages are given.

Materials tested	Alfalfa	Clover	Tomato	Spinach	Cabbage	Turnip.	Carrot	Timothy	Beet	Control
Full concentration of aqueous extract	17	21	20	17	16	14	18	14	10	2
Neutral alkaline extract	19	19	20	19	21	14	24	26	7	1
Alkaline extract with 5 cc. alkali	17	18	26	14	16	11	18	22	5	1
Half concentration of aqueous extract	16	18	18	10	14	17	16	13	8	2
Neutral alkaline extract	14	18	13	15	16	16	18	16	6	2
Alkaline extract with 5 cc. alkali	19	19	20	18	12	9	21	18	3	2
Fourth concentration of aqueous extract	13	14	14	9	10	16	15	10	5	2
Neutral alkaline extract	13	15	11	11	13	11	14	13	6	2
Alkaline extract with 5 cc. alkali	16	14	15	14	10	8	14	13	2	2
Eighth concentration of aqueous extract	11	10	10	6	7	14	13	8	5	2
Neutral alkaline extract	5	12	7	10	8	8	12	10	4	2
Alkaline extract with 5 cc. alkali	9	9	13	9	9	8	7	8	2	2
Sixteenth concentration of aqueous extract	9	8	9	5	9	9	11	4	3	2
Neutral alkaline extract	5	8	5	6	4	6	7	5	3	2
Alkaline extract with 5 cc. alkali	3	5	3	5	5	4	4	2	2	2

can justify its use as a basal diet for yeast when used to test vitamine content. We have begun the development of such experiments and a few of our preliminary results follow.

IV.

Professor McKee of the Department of Chemical Engineering of Columbia University has recently prepared two sets of carbons, one specially adapted to the adsorption of acid-reacting substances and the other to the adsorption of alkaline-reacting substances. Samples of these were furnished by him for our use. We first determined that the base-adsorbing carbon had a power of adsorption for the yeast growth-stimulating factor that was quite as good as treatment with fullers' earth (Lloyd's reagent), or precipitation with phosphotungstic acid (see Chart V). With this fact established we proceeded to treat a known concentration of alfalfa extract with this carbon. After a few hours shaking

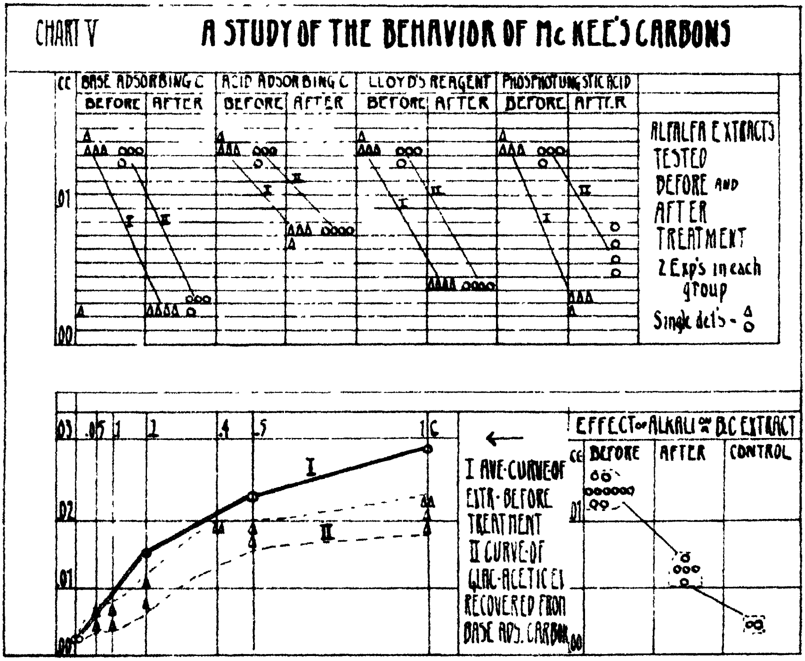


CHART V A STUDY OF THE BEHAVIOR OF McKEE'S CARBONS

and then allowing the mixture to stand for 24 hours the carbon was filtered off on a suction funnel. This carbon was then washed on the funnel and by shaking repeatedly with distilled water until all adherent material that was removable by this washing was eliminated. The washed carbon was then boiled with glacial acetic acid and the acid extract filtered off through an alundum filter. This acid extract was evaporated to dryness on the steam bath and the residue taken up with distilled water and neutralized. Its volume was made up with water to equal that of the alfalfa extract from which it was derived. This extract in full concentration and in several dilutions was tested with the yeast test and the results are shown in Chart V. When compared with the test strength of the solution from which it was obtained the recovery of the stimulation factor by this method seems to have been rather high. With a portion of this extract we repeated the alkali treatment and obtained an unmistakably destructive effect, though not complete destruction. This result leads us to retain our faith that one of the factors concerned is either vitamine B or one that resembles it somewhat closely in properties (14).

Analyses are now being conducted on the carbon recovered factor to obtain a possible clue as to its nature.

If we picture the action of a factor on a yeast cell we may assume that the process might well consist of three steps; first diffusion, by which it comes in contact with the cell; second, possibly adsorption or at any rate penetration into the cell; and finally, its specific stimulation to protoplasmic production. If this is true the following conditions will exist and influence the growth of the cell; *viz.*, number of factors present, concentration of these factors, and rate of diffusion of factor to cell. It occurred to us that the failure of high concentrations to permit optimum growth might be a matter of diffusion. The following tests were devised to test this view. The two concentrations of alfalfa extract used in the time of incubation test (Table V) were selected, and two sets of tubes prepared as usual. One set was kept perfectly quiet at room temperature, after inoculation with yeast suspension for 5 days. The second set was treated in the same way except that at intervals in the 5 days they were transferred to the shaking machine and shaken a total of 15 hours in all in the 5 days. The rate of growth in the two sets is shown in Table VIII. Having thus shown that diffusion is an important factor, a second test was devised as follows. Two sets of tubes were prepared as before but the 1 cc. of extract was in one set of tubes placed within a collodion sac suspended in the Nägeli solution. The effect of this process was to reduce slightly the growth rate but there is no doubt that the growth factor passes through the membrane; *i.e.*, is highly diffusible through membrane. The results are shown in Table VIII. We are now trying to determine whether by a study of the content of the collodion sacs before and after diffusion, light may be thrown on the nature of the factors. These few experiments are cited merely as illustrating the complexity of factors taking part in a yeast test.

TABLE VIII.

The effect of changing diffusion conditions upon the yeast test.

Shaking experiment.

	Full concentration of alfalfa.		Optimum concentration of alfalfa.	
	Test	Average	Test.	Average
5 days with no shaking......	10 11 15 11	12	22 25 22 27 22	24
5 days with a total of 15 hrs. shaking.......	22 15 21 16 26 18	20	29 35 39 34	34
Control (still)...	2 2 2	2		
Control shaken..	3 4 4	4		

Collodion bag experiment.

	Full concentration yeast autolysate extract.		Half concentration yeast autolysate extract.	
	Test.	Average.	Test	Average
1 cc. extract mixed with Nägeli solution in tube..	84 80	82	73 71	72
1 cc. extract suspended in collodion sac.........	67 62 60 82	68	64 56 61 61	60
Control..................		2		2

SUMMARY.

1. When the yeast test is applied to materials already evaluated as to vitamine content by rat-feeding experiments, the results show only approximate agreement. The agreement is more marked, however, when the extracts are dilute.

2. A study of the curve formed by plotting all the determinations obtained with varying concentrations of an aqueous extract of alfalfa shows that the reaction does not give the appearance of a monomolecular reaction. From the control point to the optimum it approximates the shape of the logarithmic curve but to the right of the optimum point there is a distinct decline indicating inhibitory factors in the higher concentrations.

3. A comparison with curves obtained from other extracts shows that the optimum growth varies not only with the concentration but with the nature of the extract tested.

4. A study of the effect of extracts upon growth of yeast cells, using Fulmer, Nelson, and Sherwood's medium F in place of Nägeli solution fails to support their contention that the growth stimulus is purely a matter of concentrations of known constituents.

5. Certain results, following the use of alkali, seem to indicate that if the solution treated is sufficiently dilute, the destructive effect of the alkali will appear; but attempts to verify this with extracts of the materials used in the rat-feeding tests gave most contradictory results. In any case it seems evident that at least some of the factors concerned in the stimulation are not affected by alkali treatment but the data is not sufficiently complete to justify the view that vitamine B is not present and therefore not one of the functioning stimulants.

6. The cumulative effect of the data obtained is to suggest that in its present state the test is distinctly unreliable as a quantitative measure of vitamine content. On the other hand it suggests interesting possibilities as a method for studying the kinds and behavior of growth stimuli.

7. Experiments are cited that illustrate some of the factors that enter into the growth results and a method of analyzing their nature is given.

8. Until a basal medium is worked out that provides an optimum of all the factors except vitamine B the test must be considered of little value in the estimation of true vitamine content.

BIBLIOGRAPHY.

1. Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.
2. Wildiers, E., *La Cellule*, 1901, xviii, 313.
3. Eddy, W. H., and Stevenson, H. C., *J. Biol. Chem.*, 1920, xliii, 295.
4. Bachmann, F. M., *J. Biol. Chem*, 1919, xxxix, 235.
5. Funk, C., and Dubin, H. E., *J. Biol. Chem.*, 1920, xliv, 487.
6. Funk, C., and Dubin, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1920, xvii, 175.
7. Souza, G. de P., and McCollum, E. V, *J. Biol. Chem.*, 1920, xliv, 113.
8. MacDonald, M. B., and McCollum, E V., *J. Biol. Chem.*, 1920–21, xlv, 307.
9. Emmett, A. D, and Luros, G. O., *J. Biol. Chem.*, 1920, xliii, 265.
10. Emmett, A. D., and Stockholm, M., *J. Biol. Chem.*, 1920, xliii, 287.
11. Fulmer, E. I., Nelson, V. E., and Sherwood, F. F., *J. Am. Chem. Soc.*, 1921, xliii, 186, 191.
12. Osborne, T. B, and Mendel, L B., *J. Biol. Chem.*, 1920, xli, 451.
13. Swoboda, F. K., *J. Biol Chem.*, 1920, xliv, 531.
14. Osborne, T. B, and Wakeman, A. J., *J. Biol Chem.*, 1920, xlii, 1.
15. Williams, R J., *J. Biol. Chem.*, 1920, xlii, 259.

NOTES ON APPARATUS USED IN DETERMINING THE RESPIRATORY EXCHANGE IN MAN.

I. AN ADAPTATION OF THE FRENCH GAS MASK FOR USE IN RESPIRATORY WORK.

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(Received for publication, May 2, 1921.)

Considerable difficulty has been experienced in finding a suitable breathing appliance to be used with respiration apparatus. To insure absolute rest and normal type of respiration it is essential that the subject suffers no discomfort. The appliance should permit him to breathe through the nose, mouth, or both as is his custom; otherwise abnormal conditions are imposed, and his attention being focused on the respiratory act, unnatural breathing results.

Appliances necessitating the insertion of tubes into the mouth have the additional defect of causing salivation, with a resulting frequence of deglutition and, in the closed circuit apparatus, the possible swallowing of oxygen. Of the appliances at present in use, the half mask used by Boothby and Sandiford[1] presents none of these defects. In practice, however, difficulties arise in the use of the half masks; the engaging surfaces are small and the bony support of the tissues deficient; in the absence of molar teeth, the cheeks sag inwards during inspiration and leaks are apt to occur. In the attempt to avoid leaks the mask is frequently applied so firmly to the face that extreme discomfort results.

The rubber gas mask used in the French army is admirably suited to this work.[2] It is made of thick rubber, covers the whole face, and presents broad surfaces which closely engage the forehead, sides of the face, and jaw. The tissues in these regions are well supported by the bony framework of the face and the mask readily adapts itself to these fixed surfaces. It is held in place by elastic straps passing around the head. In emaciated subjects, leaks may occur above or below the zygoma, in this area the pull of the straps is in the same plane as the surface of the face. In such rare instances the leaks are readily overcome by placing 6 inch rubber sponges over these areas of the mask and binding them firmly in place with a 3 inch bandage. In this mask, the incoming air is directed upwards towards the windows, the opening of the expiratory tube being opposite the nose and mouth. In this way the space is perfectly ventilated and no discomfort results. Rubber flutter valves are eminently satisfactory for use with these masks. They are conveniently enclosed in flattened glass tubes introduced as near the mask as practicable. A satisfactory arrangement of mask and valves for the Tissot method is shown in Fig. 1. The subject reclines in a wheel chair in the rest room, the tubes pass through the wall into the laboratory where the valves are mounted, the tubes then lead directly to the air supply and to the gasometer. A window permits the operator in the laboratory to observe the

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[1] Boothby, W. M., and Sandiford, I., Laboratory manual of the technic of basal metabolic rate determinations, Philadelphia and London, 1920, 35.

[2] The gas masks and valve attachments may be obtained from the C. M. Sorensen Co., 177 East 87th Street, New York.

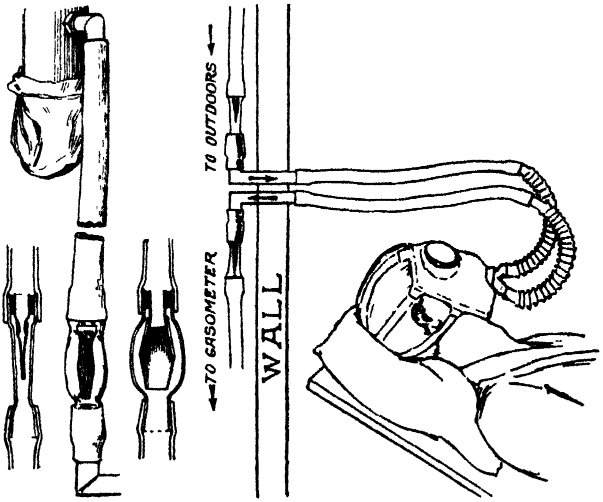

Fig. 1.

subject, while at the same time he can follow the respirations by watching the movement of the valves and start and stop the test by turning the 3-way valve on the gasometer.

The incoming air enters the room through a 4 inch pipe, the end of which is closed by a rubber bathing cap held in place by a rubber band. This takes up the pressure of gusts of wind and prevents it blowing through the valves. A 2 foot length of 24 mm. rubber tubing leads from the large pipe to the inspiratory valve, this is for the purpose of trapping any expired air which might backlash through the valve. In using the mask with closed circuit apparatus double tubes lead to the oxygen reservoir, the expiratory tube, containing a flutter valve, leads through the CO_2 absorbed. With portable apparatus the valve case can be made of transparent celluloid which is not readily broken. In this type of apparatus the use of the mask with a single tube and without valves is unsatisfactory as the dead space is increased to too great an extent.

NOTES ON APPARATUS USED IN DETERMINING THE RESPIRATORY EXCHANGE IN MAN.

II. A SAMPLING BOTTLE FOR GAS ANALYSIS.

BY CAMERON V. BAILEY.

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(Received for publication, May 2, 1921.)

This appliance is designed for readily collecting and holding samples of gas and for transferring them to a gas-analyzing burette without danger of dilution or loss of mercury.

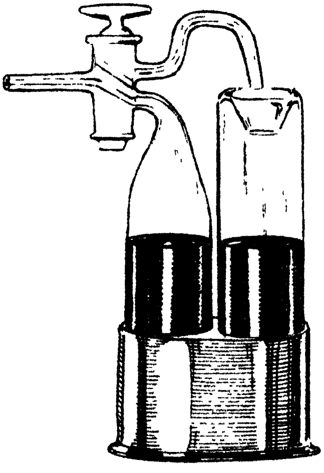

FIG. 1.

The bottle[1] shown in Fig. 1 is 19 cm. high and has a gas capacity of 60 cc.; this is a convenient size for use in conjunction with a gasometer and the Henderson[2] modification of the Haldane gas analyzer.

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[1] This bottle may be obtained from the C. M. Sorensen Co., 177 East 87th Street, New York.

[2] Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 31.

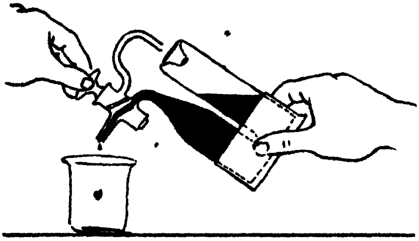

Fig. 2.

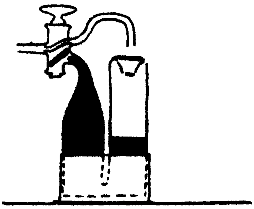

Fig. 3.

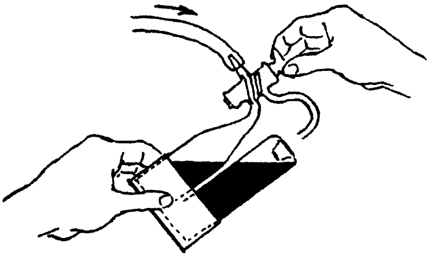

Fig. 4.

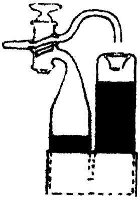

Fig. 5.

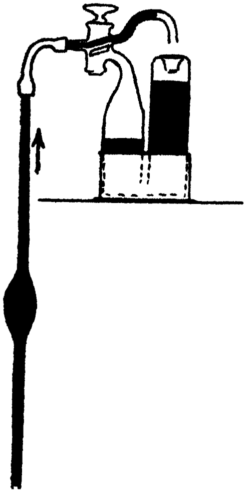

Fig. 6.

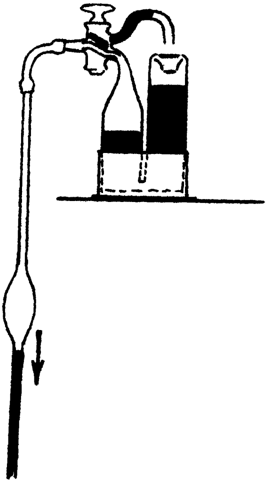

Fig. 7.

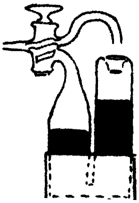

Fig. 8.

The bottle as illustrated consists of parallel glass cylinders communicating at the bottom through a small opening. The cylinders are firmly cemented into a metal case which forms a base. The top of one cylinder has a funnel-shaped inversion; the other tapers off to a 2 mm. capillary tube to which a 2-way stop-cock is fused. The second opening in the stop-cock communicates with a capillary tube which bends over the inverted top of the first cylinder. The stop-cock terminates in a straight spout of capillary tubing 3 cm. long. The appliance is half filled with mercury, and before use is flushed out with 1 per cent sulfuric acid.

The series of small drawings illustrate the manipulation of the bottle.

Fig. 2. It will be seen that by tilting the bottle forward, the mercury displaces the air in the gas compartment and in the barrel of the stop-cock.

Fig. 3. If the stop-cock be completely reversed while the bottle is in this position, these parts remain filled with mercury and the spout is in communication with the bent capillary tube through which the gas to be sampled is blown.

Fig. 4. The stop-cock is returned to its original position and the sample of gas is drawn into the bottle by tilting it backward.

Fig. 5. By turning the stop-cock while the bottle is in the last position the sample is trapped in the compartment under pressure and the spout again communicates with the curved capillary tube.

Fig. 6. The sampling bottle is now placed on a shelf overthe analyzer framework; the spout connected to the top of the gas burette and the mercury in the latter forced through this communication rendering it free from air.

Fig. 7. The stop-cock is now turned to the first position, the mercury in the burette lowered, and a sample of the gas is drawn into the gas burette.

Fig. 8. The stop-cock is again reversed and sufficient gas remains for five additional analyses.

STUDIES ON THE DIGESTIBILITY OF PROTEINS IN VITRO.

II. THE RELATIVE DIGESTIBILITY OF VARIOUS PREPARATIONS OF THE PROTEINS FROM THE CHINESE AND GEORGIA VELVET BEANS.

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The ill effects (1, 2) which have sometimes followed the use of velvet bean meal as the principal source of protein in stock feeds have lead to an investigation by Miller (1) in which he isolated a toxic substance, 3, 4-dihydroxyphenylalanine, from the seed of the Georgia velvet bean, *Stizolobium deeringianum*. This discovery seemed at the time of its publication to furnish a sufficient explanation of the objectionable properties exhibited by these beans. But feeding experiments recently made in this laboratory (3), while they tend to confirm the supposition that the velvet beans contain a toxic substance, have brought out also the interesting fact that the isolated proteins prepared from the Chinese (*Stizolobium niveum*) and Georgia velvet beans by dialyzing their saline extracts are no better tolerated than is the bean meal. There must, then, be some limiting factor other than the dihydroxyphenylalanine *alone*. Amino-acid deficiency cannot account for the failure to promote growth, for analyses (4, 5) have shown these proteins to be adequate except possibly with respect to cystine; and no improvement resulted from the addition of this amino-acid. Further, the proteins as obtained by coagulation gave normal growth. The most reasonable hypothesis to account for these observations seemed to be that the proteins in the raw state as prepared by dialysis are not sufficiently digestible to be available for animal nutrition, while the coagula were cooked, by the boiling incident to the process of preparation, enough to render them sufficiently digestible. The primary purpose of the experiments described in this paper was to test the correctness of this conclusion. We wished also to secure further data on the extent to which our results derived from experiments *in vitro* run parallel with those of growth and utilization tests made with animals.

The method employed was essentially that of Waterman and Johns (6), described in the first paper of this series. Inasmuch as proteins from different sources were to be compared in the present case, the results were calculated on the basis of the total amino nitrogen of the protein, minus the free amino nitrogen (½ the lysine nitrogen), as suggested in the first paper. The formula for the calculation of the percentage of digested nitrogen becomes, then,

$$D\ \mathrm{N} = \frac{\mathrm{N}_d - \mathrm{N}_b}{\mathrm{N}_a} \times 100 \text{ where}$$

N_d = mg. amino N found in the reaction mixture after digestion, corrected for the blank due to the reagents used in the Van Slyke amino N apparatus.

N_b = mg. amino N produced by self-digestion from the enzymes in a blank digestion; corrected as above.

N_a = mg. total amino N, derivable by complete hydrolysis from the protein, minus one-half the lysine nitrogen.

The experiments confirm our theory based upon the results of the feeding experiments, that the failure of raw dialyzed Chinese velvet bean protein was due to partial indigestibility. The figures obtained for the digestibility of the coagulated proteins of the Chinese and Georgia velvet beans are nearly twice as great as those given by the uncooked dialyzed proteins in either case, and agree well with those yielded by cooked phaseolin (6) and by casein (Table IV). The figures found for the raw dialyzed proteins are about 8 per cent lower than those given by *raw* phaseolin. This corresponds well with the growth experiments in these two cases. It seemed almost certain that the difference between the raw dialyzed and the coagulated velvet bean proteins was due simply to the cooking involved in the preparation of the latter material. There remained, however, the possibility that the coagula contained protein not precipitable by dialysis and that their greater percentage of digestible nitrogen might have been due in part to these other proteins. In order to eliminate this question we made cooked preparations of the protein obtained by dialysis from each of the two species of velvet beans and have compared their digestibility with that of the corresponding raw dialyzed proteins. Both cooked preparations showed a digestibility agreeing closely with those of the corresponding coagula.

In addition to the difference in the percentages of digestion nitrogen, certain purely qualitative indications were observed which also point to a marked superiority in digestibility of the coagulated and the cooked dialyzed preparations over those of the raw dialyzed proteins. In the first place, there appeared in the digests of the raw dialyzed samples, on neutralizing the acid after digestion with pepsin, a bulky, light gray, flocculent precipitate apparently consisting of incompletely peptonized protein. The coagulated and the cooked dialyzed protein formed only a small precipitate at this point. Also, when the digests are heated, to inactivate the enzymes' at the end of the digestion period with trypsin, a small amount of coagulation always occurs, although the solution is distinctly alkaline. In the present case this precipitate was always considerably greater in the reaction product of the raw dialyzed, than it was in those of the cooked or the coagulated proteins. Again, in making the determinations of amino nitrogen on the filtrate from these reaction mixtures, we found that the digestion products of the raw dialyzed samples foamed persistently in the Van Slyke apparatus, filling both the deaminizing bulb and the gas burette with a thick froth which made it almost impossible to carry out the analysis accurately, unless diphenyl ether or some other foam inhibitor was used. This behavior of the digests of the uncooked preparations is very characteristic of solutions containing unhydrolyzed protein. The digests of the cooked and the coagulated samples, on the other hand, gave practically no trouble of this sort. Finally, if the filtered, slightly alkaline digestion products were made just acid with acetic acid a further precipitate was produced, and, here again, its amount was considerable in the case of the raw dialyzed preparations and very slight, a mere cloud hardly greater than that yielded by the blank digestion, in the case of the cooked or the coagulated protein.

This combined evidence leaves little doubt that the difference in digestibility between the raw dialyzed and the coagulated velvet bean proteins is due simply to cooking. Also, the experiments as a whole tend decidedly to confirm our supposition that incomplete digestibility is one of the limiting factors in the nutritional failure of raw velvet bean meal and the only limiting factor in the similar failure of the protein isolated by dialysis.

EXPERIMENTAL.

Preparation and Analysis[1] *of the Proteins.*—The coagulated and the dialyzed proteins from the Chinese velvet bean were made[2] in accordance with the direction of Johns and Finks (4). The dried preparations were ground to pass a 100 mesh sieve, the powdered material was exposed to a filtered current of air to come to equilibrium with atmospheric moisture, and the total nitrogen, not corrected for ash or moisture, was determined. The total amino nitrogen of each preparation, minus its *free* amino nitrogen (½ the lysine nitrogen), was then calculated from the figures of the Van Slyke analyses given in the paper referred to above.

The coagulated Georgia velvet bean protein was prepared like that from the Chinese variety. The dialyzed protein was precipitated directly from the saline extract without preliminary fractionation by ammonium sulfate. Both of these preparations, therefore, contained a mixture of the α- and β-globulins of the Georgia bean, and in addition to these the coagulum contained the traces of albumin present in the extract. The dried mixtures were prepared for analysis and their total nitrogen determined as above described; but the presence in each of more than one protein made necessary a different procedure in the estimation of the amino nitrogen. An average of the amino nitrogen figures, taken from the nitrogen distribution given by Johns and Waterman (5) for the α- and β-globulins, corrected for free amino nitrogen as above, and weighted according to the relative yields of the two proteins, was used as the basis of the calculation in the case of the dialysis product. The maximum error which could be introduced into the calculation of the digestion nitrogen by the use of the value thus derived would not be significant for the purposes of the present experiments. The coagulum, however, contained albumin, the relative quantity of which could not be estimated with any approach to accuracy. A sample of about 3 gm. of this material was hydrolyzed, therefore, and freed from ammonia as in the determination of the distribution of nitrogen by Van Slyke's method. After expelling the ammonia, the hydrolysate was acidified with acetic acid, concentrated to a syrup, transferred to a 200 cc. graduated flask, and made up to the mark with distilled water. The amino nitrogen in this solution was then determined by means of the Van Slyke apparatus, and the N_a calculated from the value thus obtained.

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[1] The elementary analysis of the preparations was made by S. Phillips of this laboratory.

[2] This material was prepared by C. E. F. Gersdorff of this laboratory. Both varieties of the beans were furnished by the Bureau of Plant Industry, United States Department of Agriculture.

Digestion with Pepsin.—The procedure was the same as that previously described (6), except that 0.1 N hydrochloric acid was substituted for the 0.1 N sulfuric acid used in the first experiments. Two or three samples, approximately 0.500 gm., of each of the proteins to be compared were suspended each in 25 cc. of 0.1 N hydrochloric acid, 25 cc. of a 0.2 per cent solution of pepsin in the same reagent were added, and the mixtures incubated for 1½ hours at 37°. The raw dialyzed proteins from both the velvet beans readily dissolved in the dilute acid, while the coagulated and the cooked dialyzed proteins were not completely dissolved until near the end of the digestion period with pepsin.

Digestion with Trypsin.—After the peptic digestion the 50 cc. of 0.1 N acid were neutralized with 5 cc. of N sodium hydroxide and 5 cc. of a 6 per cent solution of trypsin in 0.1 N sodium hydroxide were added. The reaction mixtures were then returned to the incubator and digested for 2½ hours at 37°. The activity of the enzymes was then destroyed by heating to 80° on the steam bath, the solutions were cooled and filtered, and amino nitrogen was determined. With each set of experiments a blank digestion of the enzymes alone without any added protein was carried out in exactly the same way as were the digestions of the samples, in order that the amino nitrogen set free by the self-digestion of the enzymes might be determined and a correction made for it. The resulting data, together with the percentages of digested nitrogen calculated according to the formula given above, will be found in Tables I, II, and III. The average digested nitrogen values for casein, cooked and raw phaseolin, and for each of the six velvet bean preparations studied are presented for comparison in Table IV.

TABLE I.

Comparative Digestibility of Coagulated and Dialyzed Chinese Velvet Bean Proteins.

Preparation.	Amount of sample (protein).	Combined amino N in sample (N_a).	Amino N after digestion (N_d)	Amino N of blank digestion (N_b).	Digestion N, calculated on basis of N_a.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Coagulated.......1	501.3	51.13	48.09	18.24	58.0
2	501.8	51.18	48.59	18.24	59.1
Average...					58.6
Dialyzed.........1	502.1	54.23	30.51	18.24	22.4*
2	502.0	54.22	33.99	18.24	28.9
Average...					28.9
Coagulated.......3	500.9	51.09	47.72	19.20	55.8
4	501.2	51.12	47.72	19.20	55.8
Average...					55.8
Dialyzed.........3	501.3	54.15	35.40	19.20	29.9
4	501.2	54.14	35.90	19.20	30.8
5	501.2	54.14	35.73	19.20	30.5
Average...					30.4
General average.					*per cent*
Dialyzed...					29.7
Coagulated...					57.4

* Not included in the averages.

TABLE II.

Comparative Digestibility of Coagulated and Dialyzed Georgia Velvet Bean Proteins.

Preparation.	Amount of sample (protein).	Combined amino N in sample (N_a)	Amino N after digestion (N_d).	Amino N of blank digestion (N_b)	Digestion N, calculated on basis of N_a.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Coagulated.......1	501.0	53.96	48.56	18.80	55.2
2	501.0	53.96	48.89	18.80	55.8
Average...					55.5
Dialyzed.........1	501.5	56.02	36.50	18.80	31.6
2	501.6	56.03	36.66	18.80	31.9
Average...					31.8

TABLE III

The Effect of Cooking upon the Digestibility of Dialyzed Velvet Bean Proteins.

Preparation.	Amount of sample (protein).	Combined amino N in sample (N_a).	Amino N after digestion (N_d).	Amino N of blank digestion (N_b)	Digestion N, calculated on basis of N_a.
	mg	*mg.*	*mg*	*mg.*	*per cent*
Georgia.					
Dialyzed,					
cooked*......1	501.1	54.38	48.53	18.86	54.5
2	501.3	54.40	48.65	18.86	54.8
Average......					54.7
Georgia.					
Dialyzed, raw. 3	502.3	56.10	37.11	18.86	32.5
4	502.0	56.07	37.34	18.86	32.9
Average....					32.7
Chinese.					
Dialyzed,					
cooked*......1	501.2	52.18	48.02	17.65	58.2
2	500.6	52.12	47.62	17.65	57.5
Average....					57.9

* An aqueous suspension was boiled 2 hours, evaporated to apparent dryness on a steam bath, and ground to 100 mesh powder.

DISCUSSION.

From the properties of the velvet bean proteins brought out by these experiments we may conclude, in the absence of experimental evidence to the contrary, that our hypothesis regarding partial indigestibility as the limiting factor in the failure of the raw dialyzed Chinese velvet bean protein to promote growth is the correct one. The nutritional inadequacy of the uncooked protein, together with the fact that a normal growth rate can be secured with the coagulated protein, is then to be referred to difference in digestibility alone. The raw ground seed would have the double disadvantage of toxicity and non-available protein content, while the cooked meal would probably still contain dihydroxyphenylalanine. Our experiments also justify us in predicting with some confidence that the raw dialyzed protein from the Georgia velvet bean will behave in nutrition experiments like that from the Chinese variety; and that cooking under conditions similar to those described above will render either of these dialyzed preparations capable of supplying the protein requirement of normal growth.

TABLE IV.

Comparative Summary of Digestibility Experiments in Vitro and Nutritional Experiments in Vivo, on the Velvet Bean and Other Proteins.

Protein.	Digestibility. *in vitro* (average).	Result of growth experiments.
	per cent	
Chinese velvet bean coagulum........	57.4	Normal growth (2).
Georgia " " "	55.5	" " (2).
Chinese " " , dialyzed protein.	30.4	Supports neither growth nor life (2).
Georgia " " " "	32.3	Not tried.
" " " cooked dialyzed protein..........................	54.7	" "
Chinese velvet bean, cooked dialyzed protein.	57.9	" "
Phaseolin, raw*.........................	39.5	Does not support growth, with or without cystine (7).
Phaseolin, cooked†....................	58.7	Normal growth when supplemented with cystine (7).
Casein..................................	61.4	Normal growth.

* Recalculated from Table II, first paper (6).

† New determination, using hydrochloric in place of sulfuric acid in the pepsin digestion.

With regard to the relation between the values obtainable in determinations of the type under consideration, and the results of growth experiments with animals, the figures presented in Table IV give some indication. Six of the nine preparations listed have been fed as the sole source of protein in an otherwise adequate diet. Of this group, four produced normal growth in animals and gave relatively high digestibility values *in vitro.* Cooked phaseolin required the addition of cystine, it is true; but cystine did not improve the raw phaseolin diet, and even without the addition of cystine the cooked phaseolin maintained the weight of the animals for some time. The other two proteins tested supported neither growth nor life, and they showed a relatively low digestibility *in vitro*. Finally, the greatest digestibility so far observed in our experiments is that of casein, the principal component of the natural food of young mammals. Thus all the evidence at present available supports the assumption tentatively put forward in our first paper on this subject, that an indigestibility sufficient to interfere seriously with the capacity of a protein to furnish the amino-acid requirement of normal growth may readily be demonstrated by this method.

Concerning the relation existing between these estimations of digestibility *in vitro* and absolute digestibility as determined in the animal organism, however, we have as yet no reliable information beyond the fairly safe inference that figures for different proteins as found by the two methods may be expected to vary in the same direction and with a rough proportionality. Any direct arithmetical ratio is probably precluded by the subtraction of the considerable blank correction for the self-digestion of the enzymes in calculating the results of the estimations *in vitro*, since, of course, this self-digestion is considerably greater in the blank digestion where no substrate is present than it is in digests containing a protein sample. It is even possible that self-digestion is so far retarded by the substrate that a less error would be introduced by omitting this correction entirely than by including it in the calculation. Bayliss (8) reports an experiment in which trypsin, acting upon casein as a substrate, showed "no appreciable destruction up to eight hours." It is of interest to note in this connection that if the data of our experiments be calculated *without subtracting the blank* they yield figures much like those obtained by the absolute method in similar cases. Various experiments with casein, for instance, when calculated on this basis gave values ranging from 99.5 to 100.2 per cent. The values for the cooked and the coagulated velvet bean proteins lie between 90 and 100 per cent on this basis, and those for the raw velvet bean proteins would be about 65 to 70 per cent. The figure 73.6 has been given as the digestion coefficient for the nitrogen of velvet bean meal (beans and pods) when fed to steers (2). Until we have further experimental data bearing upon this point, however, we prefer to regard our results as purely comparative, and to include the blank in the calculation.

The chemical nature of the indigestibility of certain proteins, which manifests itself in animals by a failure to promote growth and *in vitro* by a relatively low yield of amino nitrogen set free by proteoclastic enzymes, also presents a problem of which little is known. The most probable answer to the question seems to be suggested by the work of Fischer and Abderhalden on the hydrolysis of polypeptides by proteoclastic enzymes. These investigators demonstrated that certain polypeptides are hydrolyzable by trypsin while others, containing the same amino-acid residues united in a different order, are not attacked at all. The presence of one or more of the essential amino-acids in the form of such stable combinations would, of course, explain the failure of a protein to promote growth, and would lower the yield of amino nitrogen set free from the protein by enzymes *in vitro*. A study of the undigested residue precipitable by dilute acid from the solutions remaining after our digestion experiments may throw some light upon this point; and it is intended to make such a study the basis of a future report.

SUMMARY.

Estimations of the comparative digestibility *in vitro* by the method of Waterman and Johns of six preparations of the proteins of the Chinese and Georgia velvet beans indicate: (1) That partial indigestibility is the limiting factor in the failure of raw dialyzed Chinese velvet bean protein to promote growth, and that the normal growth secured with the protein prepared by coagulation from either bean is probably to be attributed to an increase in digestibility brought about by the boiling incident to the preparation of the latter material; and (2) that cooking under the conditions described renders the dialyzed protein from either seed as digestible as the coagulated product, and that probably, therefore, these cooked dialyzed proteins will support growth as well as do the coagulated proteins. The double disadvantage of toxicity and non-assimilable protein content amply explains the behavior of the raw ground bean, while the cooked meal probably still contained dihydroxyphenylalanine.

The experiments also support the contention that the results of such estimations run parallel with the results of growth experiments, in as far as differences in the digestibility of the proteins are concerned.

BIBLIOGRAPHY.

1. Miller, E. R., *J. Biol. Chem.*, 1920, xliv, 481.
2. *U. S. Dept. Agric., Farmer's Bull. 962*, 1918, 38.
3. Finks, A. J., and Johns, C. O., *Am. J. Physiol.*, 1921, in press.
4. Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1918, xxxiv, 429. Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1919, xl, 435.
5. Johns, C. O., and Waterman, H. C., *J. Biol. Chem*, 1920, xlii, 59.
6. Waterman, H. C., and Johns, C. O., *J. Biol. Chem.*, 1921, xlvi, 9.
7. Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1920, xli, 379.
8. Bayliss, W. M., The nature of enzyme action, New York, 3rd edition, 1914, 76.

THE METABOLISM OF NITROBENZALDEHYDES AND NITROPHENYLACETALDEHYDE.

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Several investigators have shown that the metabolism of the *o*-, *m*-, and *p*-nitrobenzaldehydes is entirely different in the body of the dog from that found in the organism of the rabbit, and moreover, that the fate of the *o*-nitrobenzaldehyde is entirely different from that of the *m* and *p* compounds in the same animal organism.

We thought it of interest to determine the fate of these three compounds in the human body, and also to include in the work *p*-nitrophenylacetaldehyde, which, up to the present time, had not been employed in such experiments.

o-Nitrobenzaldehyde.

The pure substance,[1] (melting point, 44°C.), in capsules, was ingested by a man in 2 gm. doses. Because of the toxicity of the compound, only two such doses could be administered. The urine was collected, evaporated to a small volume, acidified with sulfuric acid until the urine showed an acid reaction to Congo red, and repeatedly extracted with ether; the ether was evaporated to dryness and the oily residue taken up with water. After standing 2 days, crystals were recovered from the water solution and dried in a desiccator over sulfuric acid. The melting point of the crystals, 142–144°, showed the substance to be *o*-nitrobenzoic acid. After the first 2 gm. dose of the aldehyde, 1.7 gm. of the acid were isolated from the urine, while after the second dose, 1.8 gm. were found, thus giving a yield of, respectively, 77 and 81 per cent of the substance fed.

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[1] The *o*-nitrobenzaldehyde used in this work was furnished by Dr. C. G. Derrick of the National Aniline Works.

Two doses of 2 gm. each of the *o*-nitrobenzaldehyde were fed to a dog of 10.5 kilos body weight. From the urine of the dog we isolated 75 and 80 per cent of the substance in the form of *o*-nitrobenzoic acid.

It appears, therefore, that the fate of the *o*-nitrobenzaldehyde in the human body is much the same as in the organism of the dog.

These results agree with those of Sieber and Smirnow (1), and Cohn (2), who used dogs for their experiments.

Cohn, however, when using rabbits for the same work, was unable to recover more than 10 per cent of the substance fed in the form of *o*-nitrobenzoic acid. We repeated this work on rabbits and were able to confirm his results.

o-Nitrobenzaldehyde was found to be much more toxic than the corresponding *m* and *p* compounds. A man of 70.5 kilos body weight, after receiving a 2 gm. dose showed every sign of intoxication, followed by marked albuminuria.

m-Nitrobenzaldehyde.

The same subject ingested three doses of 2 gm. each of this compound within a period of 96 hours. The urine was collected and treated as in the previous experiment. 4.21 gm. of a substance melting at 139–141° and 2.55 gm. of a substance melting at 160–162° were recovered from the urinary extract. The former substance, *m*-nitrobenzoic acid, as shown by its melting point, represented 63.51 per cent of the *m*-nitrobenzaldehyde fed, while the latter substance, *m*-nitrohippuric acid, is equal to 24.83 per cent of the substance fed, a total of 88.34 per cent.

After 6 gm. of the aldehyde were ingested by a second individual, the ratio of *m*-nitrobenzoic acid to *m*-nitrohippuric acid excreted was entirely different. In this case, about 75 per cent of the aldehyde was found in the urine as *m*-nitrobenzoic acid and only 5 per cent as *m*-nitrohippuric acid. The *m*-nitrobenzaldehyde proved entirely non-toxic, apparently causing no inconvenience to the subjects in doses as large as 5 and 6 gm.

Previous work (1) on dogs has shown that after feeding this substance, *m*-nitrobenzoic acid and *m*-nitrohippuric acid could be recovered from the urine, while after feeding to rabbits (2), an entirely different substance, *m*-acetylaminobenzoic acid was found in the urine.

p-Nitrobenzaldehyde.

This substance, in capsules, was ingested by a man in 2 gm. doses. A mixture of the corresponding benzoic and hippuric acids was obtained. Out of a total of 4 gm. taken by the subject, 62 per cent was isolated from the urine as *p*-nitrobenzoic acid and 19 per cent as *p*-nitrohippuric acid.

p-Nitrophenylacetaldehyde.

This substance, prepared according to the method of Lipp (3), by the treatment of *p*-nitrophenyl-β-chlorolactic acid with sodium carbonate, melted at 84–86°.

The *p*-nitrophenylacetaldehyde was fed to rabbits in 1gm. doses with apparently no ill effects. The urine was collected for 36 hours and treated as usual, except that extraction was first carried out with ether, followed later by two extractions of the evaporated urine with alcohol and then with ethyl acetate. From the ether extract we obtained a yellow oil on evaporating almost to dryness. This oil was difficultly soluble in hot water and after standing in the ice box for 48 hours crystallized out as yellow needles melting when dry at 151–153°. This substance was proven to be *p*-nitrophenylacetic acid by its melting point as well as by its titration value.

After feeding 4 gm. of *p*-nitrophenylacetaldehyde to rabbits, 3.86 gm. of *p*-nitrophenylacetic acid, corresponding to 88 per cent of the substance fed, were recovered from the urine. None of the substance fed, or any of its derivatives, could be found in either the ethyl acetate or alcohol extracts.

5 gm. of this substance were again fed to rabbits, 4.23 gm. of *p*-nitrophenylacetic acid, corresponding to 77.1 per cent of the substance were recovered from the urine.

5 gm. of the substance in capsules were fed to a dog and the urine treated in the same manner as the rabbit urine. From the ether extract 3.92 gm. of *p*-nitrophenylacetic acid were recovered, or 71.35 per cent of the aldehyde fed. No *p*-nitrophenaceturic acid nor any derivative of *p*-nitrophenylacetic acid could be found in either the alcohol or ethyl acetate extracts. These extracts were evaporated to dryness, the residue was redissolved in water and fractions of this solution were strongly acidified with sulfuric acid

and boiled for 2 hours. These were then cooled and extracted with ether to determine the presence of any *p*-nitrophenylacetic acid which might previously have escaped extraction in the form of an insoluble conjugation product. The absence of the acid at this point shows that no conjugation product of the acid existed in the urine and that only an oxidation of the aldehyde to an acid had resulted from the passage through the body of the dog.

A man ingested a total of 5 gm. of the *p*-nitrophenylacetaldehyde with much the same results as were previously shown for the dog and rabbit. In this case 70.13 per cent of the aldehyde was excreted in the form of *p*-nitrophenylacetic acid.

Thus, in each case where the *p*-nitrophenylacetaldehyde was fed, there was only an oxidation of the aldehyde to the carboxyl group, which is entirely comparable to the action of the three nitrobenzaldehydes; but in no case was there a reduction of the nitro group to an amino group as was found to be the case when *m*- and *p*-nitrobenzaldehydes were fed to the rabbit. There was also no conjugation of the carboxyl group with glycocoll as was seen when *m*- and *p*-nitrobenzaldehydes were fed to dogs and which was to be expected, as we (4) have previously shown that *p*-nitrophenylacetic acid when fed to dogs is to some extent combined with glycocoll and excreted as *p*-nitrophenaceturic acid.

CONCLUSION.

The fate of *o*-, *m*-, and *p*-nitrobenzaldehydes in the human body was much the same as that previously shown for the dog. In each case oxidation took place with the formation of the corresponding acid. In the case of the *o* compound about 90 per cent was excreted as the *o*-nitrobenzoic acid, while the *m* and *p* compounds were excreted to a large extent as the *m*- and *p*-nitrobenzoic acids, but also to a small extent combined with glycocoll and excreted as *m*- and *p*-nitrohippuric acids. In no case was there a reduction of the nitro group.

p-Nitrophenylacetaldehyde was fed to rabbits, dogs, and a man. In each case there was only an oxidation to the corresponding acid, with no reduction of the nitro group or combination with either glycocoll or glutamine.

BIBLIOGRAPHY.

1. Sieber, N., and Smirnow, A., *Monatsh. Chem.*, 1887, viii, 88.
2. Cohn, R., *Z. physiol. Chem.*, 1892–93, xvii, 274; 1894, xviii, 133; *Arch. exp. Path. u. Pharmakol.*, 1905, liii, 435.
3. Lipp, A., *Ber. chem. Ges.*, 1886, xix, 2643.
4. Sherwin, C. P., and Helfand, M., *J. Biol. Chem.*, 1919, xl, 17.

FAT-SOLUBLE VITAMINE.

VIII. THE FAT-SOLUBLE VITAMINE CONTENT OF PEAS IN RELATION TO THEIR PIGMENTATION.*

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(Received for publication, May 17, 1921.)

In the classification of naturally occurring foods which enter into the make-up of the human dietary the seeds were early given a position of rather uniform value. Thus in 1919 McCollum (1) makes the statement:

> "By the application of the biological method of analysis of a food-stuff to each of the more important seeds employed in the nutrition of man and animals, the fact was brought to light that they all resemble each other very closely in their dietary properties. The list of seeds examined included,—wheat, corn, rice, rolled oats, rye, barley, kaffir corn, millet seed, flaxseed, pea and both the navy and the soy bean. . . . All are, with the exception of millet seed, below the optimum in their content of the dietary factor, fat-soluble A."

From our present knowledge it is very evident that this statement merits considerable qualification, for while seeds as a class are apparently never as rich in the fat-soluble vitamine as the leafy parts of plants yet some contain considerable amounts of this vitamine, and among these millet seed, according to our investigations, does not occupy a place of special prominence. In fact, though such varieties of millet may be in existence we have never had as uniform success with these as a source of fat-soluble vitamine as we have had with yellow Indian corn.

That there are other instances where exception must be taken to this statement was brought out in the further development of our theory that the fat-soluble vitamine is biologically related to certain yellow plant pigments.

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* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

We have already shown that in Indian corn (2) those varieties carrying yellow pigment are rich in the vitamine while those free from it contain very little of this constituent. Further investigation has brought out the fact that in butter fat and in beef fat (3), while there is no absolute parallelism between vitamine and pigment content, yet probably due to their simultaneous occurrence in nature as well as resemblance in properties, where the pigmentation is high the tendency for high vitamine content obtains.

In this paper it is desired to bring out the relation of vitamine and pigment content that has been found to hold among different varieties of edible peas.

EXPERIMENTAL.

Six samples of peas were used for the comparisons. Two were purchased in the open market, the one as a sample of split peas sold for cooking purposes having been practically freed from all pericarp; the other was a small green pea of an unknown variety which is commonly used in pigeon feeding. The remaining samples were purchased from a local seed house as pure bred varieties.

Pigment estimations were made by extraction with alcohol, followed by saponification and comparison in a Duboscq colorimeter. 30 gm. of peas, finely ground, were extracted with hot 95 per cent alcohol in a Soxhlet extractor for 6 hours. The extract was then saponified over night with 10 cc. of 10 per cent alcoholic potash which had been prepared with the usual precautions. After dilution with water the alcoholic soap solution was extracted by shaking repeatedly with ether. The ether was then volatilized, the yellow pigments taken up in 40 cc. of alcohol and quantitatively compared with one another in the Duboscq colorimeter. The results of this comparison are shown in Table I.

The analysis for relative vitamine concentration was made by the use of the rat as the experimental animal following the technique described in previous publications. Four animals were fed in each group, one group being used for each sample. The results as presented in Charts I and II show the complete data obtained with the various groups when fed peas at a 15 per cent level as the sole source of the fat-soluble vitamine in their diet.

TABLE I.

Relative Yellow Pigment Content of Green and Yellow Peas.

Color	Designation	Relative pigment values
Yellow.	Commercial split.	39
"	Marrowfat.	44
"	Canadian field.	55
Green.	Small green.	76
"	Alaska.	100
"	Scotch beauty.	95

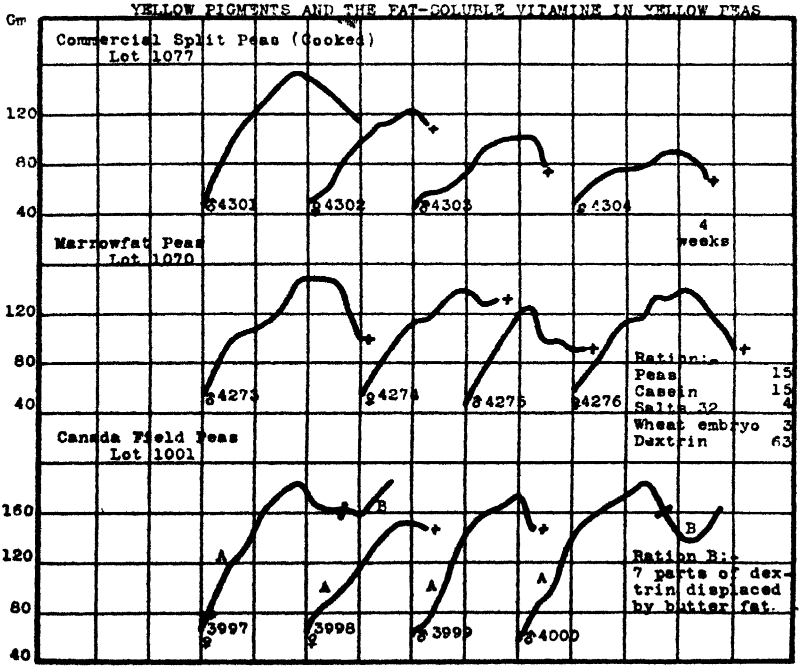

CHART 1.

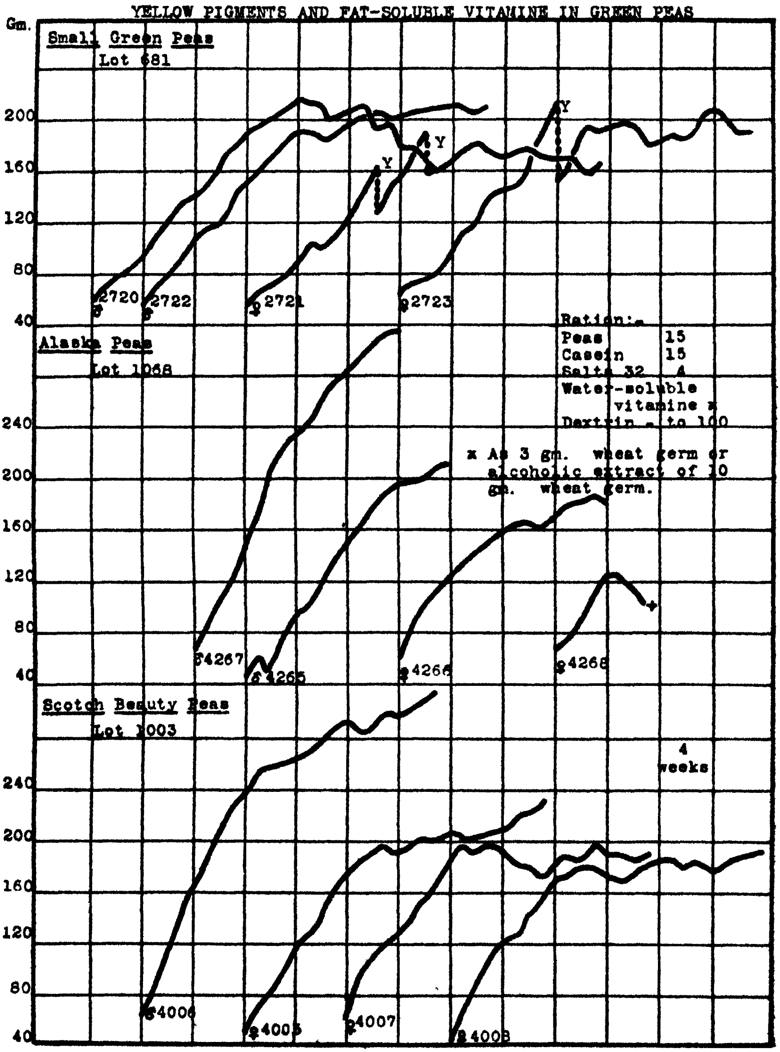
YELLOW PIGMENTS AND FAT-SOLUBLE VITAMINE IN GREEN PEAS
Gm.
Small Green Peas
Lot 681
200
160
120
80
40
2720
2722
2721
2723
Y
Y
Y
Ration:-
Peas 15
Casein 15
Salts 32 4
Water-soluble vitamine x
Dextrin - to 100
x As 3 gm. wheat germ or alcoholic extract of 10 gm. wheat germ.
Alaska Peas
Lot 1068
240
200
160
120
80
40
4267
4265
4266
4268
Scotch Beauty Peas
Lot 1003
4 weeks
240
200
160
120
80
40
4006
4005
4007
4008

Chart 2.

In Chart I are shown the growth curves resulting when *yellow* peas were used as the medium for the introduction of the fat-soluble vitamine in the diet. In view of the abundance of hemicellulose found in peas it was surmised that the peas would have to be cooked to avoid causing digestive disturbances. This was done with the commercial split peas fed to Lot 1077 but not with any of the other samples as in the course of other feeding trials with peas it was found to be unnecessary although tympanites was occasionally observed. All the animals in the three lots contracted eye infections and four developed respiratory trouble in addition. In Lot 1001 two of the rats; *viz.*, Rats 3997 and 4000 responded gradually when butter fat was added to their ration. With the others no improvement of ration was attempted, yet from the consistency of the data as a whole no doubt is left that failure of growth and maintenance was due to a fat-soluble vitamine deficiency.

That yellow peas are not to be classed with seeds of very low fat-soluble vitamine concentration, such as wheat, oats, and barley, is shown by the very appreciable growth which occurred during the first 2 months on the experimental ration. On the basal ration without supplementation with peas young rats of the age used in these experiments rarely grow longer than 6 weeks.

Further experimentation with the amount of peas increased to 50 per cent of the ration gave more conclusive evidence of an appreciable fat-soluble vitamine content. On the marrowfat peas one rat with an initial weight of 60 gm. rose to 285 gm. after 4 months subsistence on the ration; yet its intake of the fat-soluble vitamine was not sufficient, as 4 weeks later, it as well as two others of this group, died from respiratory infections at an approximate weight of 170 gm.

The results of feeding green peas are shown in Chart II. Though fed at the same level as the yellow peas; *viz.*, 15 per cent, the curves of growth in themselves show their superiority as a source of the fat-soluble vitamine. In these three groups by way of contrast there was observed only one instance of suggested incipient ophthalmia and one instance of respiratory infections which occurred, respectively, in Rats 4267 and 4265. In the complete failure occurring with Rat 4268 the cause of death was obviously of a foreign nature as no symptoms indicating fat-soluble vitamine were observed.

SUMMARY.

In ripe peas, out of six samples investigated, those of a green color, also carrying considerable yellow pigment, were far richer in their fat-soluble vitamine content than yellow peas which contained much less yellow pigment.

BIBLIOGRAPHY.

1. McCollum, E. V., The newer knowledge of nutrition, New York, 1919, 38.
2. Steenbock, H., and Boutwell, P. W., *J. Biol Chem*, 1920, xli, 81.
3. Steenbock, H., Sell, M. T, and Buell, M. V., *J. Biol. Chem*, 1921, xlvii, 89.

THE DETERMINATION OF CRESOL BY THE PHENOL REAGENT OF FOLIN AND DENIS.

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(Received for publication, May 9, 1921.)

At first sight the colorimetric method of Folin and Denis[1] promised to dispel most of the difficulties and uncertainties previously attending the determination of phenols in low concentrations. They and others immediately applied it to urine, feces, blood, etc., with apparently gratifying results. But a critical survey of the literature reveals that too little of the relatively unattractive foundation work has been executed. The temptation has been to utilize the method for the solution of certain problems without taking time for close scrutiny of its reliability under the operating conditions.

That a rather large number and wide variety of substances may develop color with the reagent has been shown by Gortner and Holm[2] and by Levine.[3] It may be doubted whether adequate measures have been taken to exclude non-phenolic substances in some reported work. But even if the color developed has been in all cases entirely due to "phenols," very little attention has been given to fundamental quantitative relations. For example, Folin and Denis determined the phenolic substances in urine as if they were exclusively phenol even though they state that "the most important phenol quantitatively is in fact paracresol."

The phenol reagent diluted with water yields a clear yellow solution. If there be added an excess of alkali or of an alkaline salt, even sodium acetate, the yellow color fades at a rate proportional to the degree of alkalinity. The final result is a colorless liquid, provided no traces of reducing impurities have gained access. Slightly alkaline mixtures remain clear; strongly alkaline ones soon deposit a considerable quantity of a white precipitate. The loss of yellow color marks the degradation of the active substance in the reagent, for at the end it no longer affords color with either phenol or sodium sulfite. If a phenol be present when the neutralizing agent is added the primary product of the reaction with the phenol is green. The green will change to blue at a rate proportional to the alkalinity and the temperature. The nature of the phenol is also of considerable influence. The primary products from *m*-cresol and *p*-cresol change color most readily, followed by that from phenol, while that from *o*-cresol lags far behind. The change in all cases is effected completely and quickly by sodium sulfite, as noted by Benedict and Theis.[4] Excessive alkali diminishes the intensity and permanence of the color, but in varying degree with different phenols.

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[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305, 309; 1916, xxvi, 507.

[2] Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1920, xlii, 1678.

[3] Levine, V. C., *Science*, 1920, lii, 612.

If it is merely a matter of measuring a single phenol against the same phenol as a standard, no very strict regulation of conditions may be necessary, provided they be maintained closely parallel in sample and standard. But when a phenol or a mixture of phenols is to be measured against an entirely different standard phenol then it becomes important to select the set of conditions which will yield the most uniform and dependable results.

EXPERIMENTAL.

Assuming a certain appropriate weight of a given phenol in a certain final volume, it is necessary to determine (*a*) the proper quantity of reagent, (*b*) the proper nature, quantity, and reaction period of the alkali, (*c*) the effect of developing or stabilizing agents, and (*d*) the color factor for the phenol against a chosen standard phenol under the conditions finally selected as standard.

Stock solutions were prepared by weight from pure dry samples of phenol and the three cresols. These were freshly diluted for use and such aliquots were introduced into 100 cc. volumetric flasks as would yield colors closely approximating that yielded by 0.5 mg. of phenol under the same conditions of treatment, which constituted the standard. All the work was done at room temperature, 22–29°C. Color comparisons were made in the Duboscq colorimeter, the standard being set at 20 and first read against itself as recommended by Folin and Denis.

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[4] Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1918, xxxvi, 95.

Folin and Denis bettered their original reagent in accordance with a formula attributed to Bell. Wu[5] has made some further improvements, and his formula has been used in the present work. Preliminary experiments showed 3 cc. of reagent to be ample for the development of maximum color.

The alkali previously used has been sodium carbonate. As Experiment 1 will show, sodium bicarbonate yields heavier colors and more consistent factors and is sufficiently rapid in action. The mixture of each phenol with the reagent was diluted to 63 cc., 5 gm. of pure powdered $NaHCO_3$ were added and the flask was swirled until solution was complete (about 2 minutes).

The colors were developed by the addition of 5 cc. of a 10 per cent solution of anhydrous sodium sulfite. Benedict and Theis merely state that sulfite is to be added "after the sodium carbonate." But since sulfite itself will reduce the reagent with production of a deep blue (Wu), it obviously must not be added until sufficient time has elapsed to insure complete inactivation of excess reagent. Experiments showed that in absence of phenol complete inactivation was effected by 5 gm. of $NaHCO_3$ in 20 minutes. Therefore, all tests were allowed to stand one-half hour after solution of bicarbonate before sulfite was added. The contents of the flasks were then made to volume, mixed, and filtered, the first 25 cc. of the filtrate being discarded. Filtration may not be necessary under ordinary circumstances, for the only undissolved matter present appears to arise from traces of impurities in the reagent. But if filtration is omitted the flasks should be allowed to stand 15 minutes after addition of sulfite to insure sufficient time for its action. Colorimetric comparisons were executed immediately after filtration. The colors are probably fairly stable, but even so, the colorimetric comparison is so simple and rapid a process that a cogent excuse for delaying it is hardly likely to arise.

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[5] Wu, H., *J. Biol. Chem.*, 1920, xliii, 189.

Experiment 1.—In comparison with the method above described as standard, tests were run in which 10 cc. of a solution of sodium carbonate of specific gravity 1.15 (16 gm. of Na_2CO_3 per 100 cc.) were employed in place of sodium bicarbonate. The colors obtained were approximately the following fractions of the colors yielded by the standard method: Phenol, 0.90; *o*-cresol, 0.78; *m*-cresol, 0.83; *p*-cresol, 0.79. That is, a given weight of *o*-cresol, for example, yielded only 78 per cent as deep a color with sodium carbonate as it yielded with sodium bicarbonate. It is evident that the cresols are affected in different degree from phenol.

TABLE I

Factors $\frac{\text{Weight of cresol.}}{\text{Weight of phenol.}}$

	Weight of *o*-cresol	Weight of *m*-cresol	Weight of *p*-cresol	Corrected readings	Average factor.
	mg.	*mg.*	*mg.*		
Series A. Temperature, 22–24°. Phenol, 0.532 mg.	0.595			21.0	
				21.2	1.181
		0.633		18.2	
				18.0	1.077
			0.648	20.2	
				20.1	1.227
Series B. Temperature, 28–29°. Phenol, 0.435 mg. . .	0.526			19.6	
				19.9	1.193
		0.456		20.7	
				21.3	1.101
			0.529	19.6	
				20.1	1.207
Final average factors	1.19	1.09	1.22		

Experiment 2.—In determining the color factor for each cresol by the standard method, two independent series of stock solutions were prepared, and two independent runs were made on each series. The results are reported in Table I. Each reading given is the average of at least three readings actually taken, and is corrected to be comparable to the standard phenol set at 20 on the same scale.

The theoretical factor $\frac{\textit{molecular weight cresol}}{\textit{molecular weight phenol}}$ is 1.15. Taking commercial cresol as a 35:40:25 mixture of the isomers, the empirical factor would be 1.16.

Experiment 3.—To investigate the factor for xylenol, solutions were prepared from selected clean and colorless crystals of purchased material. Only one test was made. The factors were as follows: *o*-xylenol (1:2:4), 1.63; *p*-xylenol (1:4:2), 1.29. The theoretical molecular factor is 1.30.

It appears, therefore, that if the composition of the cresol be known to the extent that it is either predominantly a certain one of the isomers or the ordinary commercial mixture, and the proper indicated factor be employed, the results need not be in error by more than 1 part in 20.

The method has shown considerable practical value in this laboratory for the determination of phenolic preservatives in serums, etc. Whenever a sample of such a product has evinced abnormal physical or biologic characteristics it has here become customary to run a determination for its content of preservative. Elvove[6] distilled the highly diluted serum from a large flask to minimize interference by foaming, and applied Millon's reagent to the distillate. For a number of reasons the present phenol reagent is preferable to Millon's reagent. The writer has not attempted to dispense with distillation, for it has been necessary that the results reported should be free from suspicion. But it has been found that silicotungstic acid[7] is an effective precipitant for the substances which cause foaming, and therefore enables the distillation to be conducted more conveniently. To 1 cc. of serum in a 300 cc. flask are added 125 cc. of water, 4 cc. of 1:3 (by volume) H_2SO_4, 4 cc. of a 12 per cent solution of silicotungstic acid, and a fragment or two of hot pumice. The flask is connected to a nearly vertical condenser by a three-bend tube. The contents are slowly brought to boiling, and the distillate is received in a 200 cc. volumetric flask. When the latter is half full, *i. e.* when 100 cc. has been distilled off, the flame is withdrawn and 100 cc. more water are added to the distillation flask, then distillation is resumed and continued until the receiving flask is filled nearly to the mark. Of the distillate, made to the mark and mixed, either 20 or 50 cc., depending on whether phenol or cresol is the preservative probably used, are brought into a

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[6] Elvove, E., *Bull. Hyg. Lab., U. S. P. H., No. 110,* 1917, 25.

[7] This substance is now rather extensively used in alkaloidal work, particularly for the determination of nicotine, and should be procurable without difficulty.

100 cc. flask and the determination is executed according to the standard method previously given. Readings on the colorimeter should naturally be not too far from 20. Otherwise the more strongly colored solution should be properly diluted before comparison. Again, the latter procedure should be not trusted to too great an extent so that it may be necessary to repeat the determination on a more appropriate volume of distillate. If it is further necessary to distinguish between phenol and cresol the method previously described by the writer[8] may be utilized as a qualitative test. Into a test-tube are brought 10 cc. of the distillate, and in a similar tube a colorimetrically equivalent quantity of the standard phenol, diluted to 10 cc. To each tube are added 5 cc. of the special Millon reagent, and the tubes are heated as described. A comparison of the resulting colors in the tubes will plainly reveal whether the preservative employed has been essentially phenol or cresol.

SUMMARY.

It is shown that the quality and intensity of color developed by a phenol with the reagent of Folin and Denis is dependent both upon the nature of the phenol and the composition of the medium. The conditions affording colors of maximum intensity and most consistently comparable when developed from differing phenols have been determined. Accurate quantitative comparison between different phenols demands an empirical factor.

By use of prescribed empirical factors and adherence to standard working conditions, the cresols may be determined with fair accuracy as "total cresol."

The determination of phenolic preservative in serums is described.

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[8] Chapin, R. M., *J. Ind. and Eng. Chem.*, 1920, xii, 771.

THE EFFECT OF HYDROCHLORIC ACID INGESTION UPON THE COMPOSITION OF THE URINE IN MAN.

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(Received for publication, May 12, 1921.)

The data presented herewith are in a way an extension of an experiment reported in 1915 which concerned the effect of the administration of hydrochloric acid on the excretion of Na, K, Mg, and NH_3 in the urine and feces of dogs (1). Increases in the amounts of all these substances were noted in the urine. The augmentation was especially noteworthy in the case of ammonia and almost negligible in the cases of calcium and magnesium. However, the data showed the calcium and magnesium content of the feces to be increased while sodium and potassium were unaffected.

Not long after the above results were published a paper by Givens (2) appeared in which the author concluded that his data failed to demonstrate any unequivocal effect of hydrochloric acid on the calcium and magnesium content of the feces of dogs. In view of the conflicting data it must be admitted that the question is still an open one but an examination of the respective sets of data will show that those of Givens show considerable lack of evenness.

In Table II on Diet B, Dog M does show a higher Ca excretion during the acid period than during the control period but instead of making the obvious interpretation that the acid was the cause Givens offers the explanation that "it seems more plausible to assume that in the fore period . . . . the animal was trying to store up some lime after its depletion by the former low lime régime." "Former" refers to a previous experimental period. Neither is it at all obvious why, in the same table, the excretion of Ca on the highest Ca diet falls short of the quantity excreted during the preceding acid period. This applies to the experiment with Dog N as well, but in this instance the Ca excretion during the period of highest Ca intake is even lower than that in a period of lower lime intake.

The problem as far as the dog is concerned has some rather difficult aspects. In order to overcome the difficulty resulting from the impossibility of separating the feces of the experimental periods sharply it is necessary to have long experimental periods. The diet must, of course, be uniform throughout the experiment and consequently of a nature so attractive that it will at no time be refused. As far as the dog is concerned this means that meat must be the principal constituent. Meat, however, is well suited to defeat the object desired since the ammonia resulting from its metabolism at once becomes available for the neutralization of any acid administered. Another possible difficulty presents itself in trying to determine where any bases excreted in response to acid administration will be found. It is not necessarily true that they need be in the feces corresponding to the day on which the acid was administered. Conceivably, they may be found in the feces of an earlier day depending upon what part of the alimentary tract is concerned with the excretion.

The present communication deals with the effect of hydrochloric acid ingestion upon the urinary composition in man. Advantage was taken of the experience gained in the earlier experiments and the diet so arranged as to make any variations as prominent as possible. It was ample calorically, but low in protein and sodium chloride. The components were rice, French fried potatoes, bananas, wheat bread, jam, butter, apple pie, and figs. None of the food was salted. The quantities were as uniform from day to day as careful measurement could make them and the only variation throughout the 7 days the diet was adhered to consisted in the ingestion of 3.65 gm. of hydrochloric acid in the form of tenth normal acid daily during the last 3 days. It was divided into three approximately equal portions and was drunk just before meals.

Analytical Methods.

For the estimation of Ca and Mg the procedure developed by McCrudden (3) was employed. Na and K were first isolated as the mixed sulfates and the potassium was then separated as the cobaltic nitrite compound. The yellow precipitate was then oxidized with a standard permanganate solution. The details of the process were those described by Drushel (4). Ammonia was determined by aeration, chlorides by the Volhard-Arnold method, and phosphates by titration with uranium acetate. The hydrogen ion concentration was measured electrometrically.

TABLE I.

Individual	Day of diet.	Total N.	Chlorides as NaCl	NH_3	Na as NaCl.	K as KCl	Ca	Mg	H_3PO_4	Ph
A. C. M.	3	6 85	4 22	0.294	3 56	4.61	0 058	0.104	1.11	6 70
	4	6.59	5.70	0.256	2.55	3.80	0 075	0 115	1.18	6.75
	5	7.95	10.47	0.559	5 19	8 74	0.090	0 181	1.64	6 28
	6	7.34	9.36	0 663	4.09	6.26	0.090	0 159	1.74	5 27
	7	6.97	11.69	0.731	4.11	7.17	0 113	0.161	1.69	5.05
R. L. S.	3	6.27	4.38	0 399	3 60	2.70	0 072	0 106	0 85	5.86
	4	6.16	5.03	0.300	3.70	4.10	0 100	0 094	0 97	6 44
	5	6.42	8.73	0.515	4.60	5.26	0.141	0.142	1 27	5.89
	6	6.29	9.76	0 730	4.29	5.96	0.123	0.135	1 53	5.05
	7	6.29	9.63	0 758	3.29	5.90	0 171	0 122	1.64	5.00

TABLE II.

	A. C. M.	R. L. S.
NaCl equivalent of increased NH_3 excretion........	1.294	1.091
NaCl " " " Na "	1.41	0.41
NaCl " " " K "	2 49	1.81
NaCl " " " Ca "	0.090	0.172
NaCl " " " Mg "	0.282	0.161
NaCl " " " H^+ "	0.340	0.271
Total..	5.906	3.915

The effect of the acid on some of the urinary constituents was as given in Table I.

The sodium chloride equivalent of the 3.65 gm. of hydrochloric acid taken daily is 5.83 gm. If the sodium chloride equivalents of the increased NH_3, Na, K, Ca, Mg, and H^+ concentrations are calculated the results are as given in Table II.

The changes in H ion concentration are taken into account here for the following reason. The pH of the foreperiod shows that there must have been practically equal quantities of primary and secondary phosphates present. The pH of the acid period shows that practically all of the phosphate must have been present in the primary form. Consequently, while the conversion of the secondary phosphate into primary does not involve any change in the amount of phosphate excreted it does signify that a portion of the hydrochloric acid has been neutralized and must be taken into account in the attempt to account for the hydrochloric acid ingested.

If it be assumed that the increased phosphoric acid excretion is due to the conversion of disodium phosphate of the blood and tissues into monosodium phosphate, then for every atom of sodium excreted as sodium chloride which came from the disodium phosphate in question there will have been excreted an extra atom of sodium in the form of monosodium phosphate. Hence from the sum of the sodium chloride increases given above one atom of sodium for every extra molecule of phosphoric acid must be subtracted.

	A. C. M.	R. L. S.
NaCl equivalent of increased NH_3, Na, K, Ca, Mg, and H^+ excretion	5.906	3.915
Na excreted as NaH_2PO_4 and not indicative of acid neutralization	0.328	0.340
NaCl equivalent of acid neutralizing factors	5.578	3.575

In the case of A. C. M. 96 per cent of the ingested acid is accounted for while in the case of R. L. S. the fate of only 61 per cent is apparent. Both of these percentages are undoubtedly influenced by physiological factors, the control of which was impossible, and it is probably true that the mean of the two would approach the true average value more closely than does either of them alone. This would leave about 22 per cent of the acid to be accounted for. The results of similar experiments on the dog mentioned at the beginning indicate that increased excretion of calcium and magnesium by the intestine may be a factor, but no data are available in the present instance to indicate the extent to which this may be true.

If the bases of the body are drawn upon in any condition of acidosis in the same way that they have been drawn upon by the hydrochloric acid in the present experiments then it is interesting to see how the drain could be met to the best advantage. A simple calculation based upon the figures of A. C. M. in Table II shows that for every 3.65 gm. of hydrochloric acid (which is equivalent to 10.4 gm. of β-hydroxybutyric acid) there would be required in order to balance the increased NH_3 formation and to meet the loss of K, Na, and H_3PO_4, 3.70 gm. of $KHCO_3$, 0.98 gm. of K_2HPO_4, and 3.88 gm. of $NaHCO_3$.

The calcium excretion *via* the urine is affected to a negligible extent but if the fecal excretion is affected in man to the same extent that it is in the dog as shown in the experiment previously referred to (1) then the above materials should be supplemented by a small quantity of some suitable calcium compound. It may be, however, that under conditions of alkali administration the effect on calcium excretion would be of no consequence.

CONCLUSIONS.

Data are presented which show that the ingestion of hydrochloric acid causes an increased excretion of potassium, sodium, ammonia, phosphoric acid, and hydrogen ions.

BIBLIOGRAPHY.

1. Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.
2. Givens, M. H., *J. Biol. Chem.*, 1918, xxxv, 241.
3. McCrudden, F. H., *J. Biol. Chem.*, 1909–10, vii, 83.
4. Drushel, W. A., *Am. J. Sc.*, 1908, xxvi, series 4, 555.

THE DISTRIBUTION OF CALCIUM AND PHOSPHORIC ACID IN THE BLOOD OF NORMAL CHILDREN.*

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The acid-base equilibrium of the body is a subject which has been much discussed, and concerning which many theories have been advanced. A review of the literature reveals a large volume of uncorrelated data, observations having been made on one or another constituent of the blood or urine under conditions which were in no way comparable. So far as we are aware no studies have been made on the distribution of calcium and the various compounds of phosphoric acid in corpuscles and plasma on the same sample of blood. It seemed to us that a knowledge of the relation of these substances to each other might throw some light on the mechanism of acid-base regulation which would be of value in certain pathological conditions. This paper is a report on a series of observations made on the blood of normal children ranging in age from 4 weeks to 14 years which we shall use as a basis of comparison for other work now in progress.

Technique.

All the children under 3 years of age and a few of the older ones were from the wards of the University of California Hospital.[1] They were either surgical or convalescent cases and presumably had normal metabolism. The others were secured from a nearby orphanage and were apparently in good physical condition.

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Collection of Blood.—The blood of children over 18 months of age was collected before breakfast, about 15 hours after the last meal. That of children under 18 months was collected about 11 hours after the last feeding. 1 drop of a saturated solution of sodium citrate to every 5 cc. of blood was used to prevent coagulation. When possible, the blood was taken from the median arm vein. In the younger children it was usually taken from the external jugular, and in babies from the longitudinal sinus. From 25 to 30 cc. were drawn through a hollow needle into a clean, dry syringe, and of this, about 15 cc. were introduced by means of a short piece of glass tubing into a graduated centrifuge tube containing 3 drops of the citrate solution and 1 cc. of paraffin oil. Care was exercised to introduce the blood beneath the surface of the oil so as to avoid a possible exchange of acids and bases between the corpuscles and plasma. The remainder of the blood was quickly introduced into a test-tube containing 2 or 3 drops of the citrate solution and was used for the determinations on whole blood.

Hematocrit Determinations.—Immediately after the collection of the blood the plasma and corpuscles were separated by centrifugation at about 4,000 revolutions per minute for 20 minutes and the hematocrit reading taken. In order to secure accurate values it was found necessary to recalibrate all our centrifuge tubes. After removing the oil and plasma as completely as possible, the corpuscles were washed with 0.9 per cent NaCl solution and centrifugated as before The volume of cells was again noted to insure a constant reading. Sundstroem and Bloor (1) suggest the use of a capillary tube for determining the percentage of cells, claiming that the readings taken in this way differ from those obtained in the graduated centrifuge tube. We found this to be true. Since, however, our hematocrit determinations were used primarily to check our work by computing the values in whole blood from those found in corpuscles and plasma, we necessarily used the readings of the centrifuge tube.

Dilutions.—In order to economize in material as well as time, we made dilutions which could be used for both the calcium and phosphoric acid determinations. The following were found satisfactory: (1) About 7 cc. of well mixed whole blood were laked with an equal volume of distilled water; (2) 3 cc. of corpuscles were laked with 9 cc. of distilled water; and (3) about 6 cc. of plasma were diluted with an equal volume of distilled water.

Phosphoric Acid Determinations.—Bloor's (2) nephelometric method with a few minor changes was used. Determinations were made on total, lipoid, and inorganic phosphoric acids in whole blood, corpuscles, and plasma. So called "other forms" of phosphoric acid were calculated by subtracting the sum of the lipoid and inorganic values from the total. The whole blood determinations were made merely as a check on our work, and in every case agreed with the calculated value within the limits of experimental error.

For the determination of lipoid phosphoric acid, 3 cc. of each of the above dilutions of whole blood (1:1), corpuscles (1:3), and plasma (1:1) were used. The solutions were added drop by drop to the alcohol-ether mixture in 25 cc. volumetric flasks and treated as directed by Bloor. Since the phosphoric acid content of the extract was twice as great as that of Bloor's, 5 cc. of the filtrate were used instead of 10 cc. In this way there was not only a large saving in alcohol and ether, but the evaporation of the smaller volume of liquid required much less time.

For the determination of inorganic phosphoric acid in whole blood, corpuscles, and plasma, 1 cc. of each of the dilutions as given above was added dropwise to 9 cc. of the acid ammonium sulfate solution and treated as directed.

For the determination of total phosphoric acid the dilutions were as follows: (1) 3 cc. of the 1:1 dilution of whole blood, made up to 10 cc.; (2) 1 cc. of the 1:3 dilution of corpuscles, made up to 5 cc.; and (3) plasma, 1:1 dilution.

1 cc. of each of the above dilutions was used. The solutions were introduced into Pyrex digestion tubes (1 by 10 inches) and evaporated to dryness on a water bath. This obviated the necessity of using glass beads during the digestion. Ordinary Pyrex tubes were found unsatisfactory, the strong acid mixture very quickly eating out the bottom and probably introducing an error into the determination. We succeeded in having satisfactory tubes made out of a special grade of Pyrex tubing. Instead of using the microburners with an improvised fume absorber as suggested by Bloor, we supported our tubes on wire gauze in the Kjeldahl apparatus where a large number of digestions could be carried on at one time.

We found it convenient to digest all the total and lipoid phosphates (six tubes) at the same time and to carry them through the various steps together, using only one standard (0.006 mg. H_3PO_4 per cc.), as a rule, for the nephelometric readings. For the inorganic phosphoric acid a standard containing 0.006 mg. of H_3PO_4 was also used. In starting our work we experienced considerable difficulty on account of a precipitate which flocked out of the strychnine-molybdate solution when no phosphoric acid was present. This was found to be due to an insufficiency of strychnine sulfate for the amount of sodium molybdate used. No further difficulties were encountered and the remainder of the procedure was as given by Bloor. In order to secure the greatest accuracy possible we recalibrated all our pipettes and volumetric flasks and used only one pipette of each volume throughout the series. In this way our actual and calculated values were in remarkably close agreement.

Calcium Determinations.—Lyman's (3) nephelometric method with a few modifications was used. 5 cc. of the whole blood (1:1), corpuscle (1:3), and plasma (1:1) dilutions were added drop by drop to 20 cc. of the trichloroacetic acid solution in 100 cc. Erlenmeyer flasks, filtered, and 10 cc. of each filtrate used for the determinations. If care is exercised, two 10 cc. portions can be obtained from each filtrate, enabling one to run duplicates if necessary. In using this method it is essential that the reactions be just right, else the calcium is not precipitated. To insure this we standardized our solutions and determined the end-points with utmost care, using alizarin rather than methyl orange for an indicator. Instead of shaking the solutions for 10 minutes as directed by Lyman, we allowed ours to stand in the refrigerator over night. By using sharply pointed centrifuge tubes and decanting the supernatant liquid quickly, we got better results than by pipetting the liquid from less pointed tubes. As was done in the case of phosphoric acid, the whole blood, corpuscle, and plasma solutions were carried through the various steps together. A standard containing 0.1 mg. of calcium was used, and was invariably checked against itself before the readings on the unknown solutions were taken.

In order to determine the accuracy of the method, known quantities of calcium were added to blood and blank solutions, and in all cases were recovered quantitatively. In addition, many determinations which seemed extraordinarily high were checked by means of a potassium permanganate titration method, the principle of which was essentially the same as that of Lyman's except that the precipitate of calcium oxalate was dissolved in N sulfuric acid and titrated with 0.01 N permanganate solution. After making determinations on about twenty samples of blood with both methods, we finally adopted Lyman's, since, with the titration method, our whole blood, corpuscle, and plasma readings frequently failed to check, while with the latter, our actual and calculated values practically always agreed within the limits of experimental error. We attribute the lack of agreement between the results secured with the former method to the fact that the end-point with 0.01 N potassium permanganate is very difficult to obtain, and 1 drop too much or too little may introduce an error of 20 per cent in the final value, the percentage of course, depending upon the volume of permanganate used in the titration.

Alkali Reserve.—The CO_2-combining power of plasma was determined by the Van Slyke-Cullen (4) method without change.

The results of the analyses are given in Tables I and II and are expressed in mg. of calcium and phosphoric acid per 100 cc. of whole blood, corpuscles, and plasma. Certain values which seemed exceptionally high were not included in the average. To be consistent, if the value of a constituent was not included in the average for corpuscles, the values of that same constituent were omitted from the averages for whole blood and plasma.

DISCUSSION.

The distribution of calcium and the various compounds of phosphoric acid was studied in thirty-four normal children, seventeen boys and seventeen girls. In general, the values for phosphoric acid are comparable to those reported by Bloor (5) and others. The total phosphoric acid values of corpuscles averaged 257 mg. per 100 cc. in boys, and 255 mg. in girls as compared with Bloor's values of 248 mg. for men and 249 mg. for women. On the whole, the averages for boys tend to run higher than those for girls. This is especially striking in the lipoid phosphoric acid content of the corpuscles, the average for boys being 65.7 mg. per 100 cc. with variations from 36 to 84, and for girls, 55.8 mg. with variations from 33 to 72. Bloor's averages were 57.0 and 56.6 mg. for men and women, respectively. Another striking difference between the values reported for adults and children is found in the inorganic phosphoric acid content of corpuscles. For men, Bloor found an average of 18.7 mg. per 100 cc. and for women, 15.7 mg. Our values for boys ranged from 6.5 to 20.9 with an average of 12.1, while those for girls varied from 5.7 to 26.0 mg. with an average of 10.3. The inorganic phosphoric acid content shows a greater percentage variation than that of any phosphorus compound in the corpuscles, or approximately 221 per cent in boys and 356 per cent in girls.

In the plasma the higher values of the total and lipoid phosphoric acids are again noted in the boys. The total phosphoric acid content varied from 33 to 48 mg. in boys with an average of

TABLE I.

The Distribution of Calcium and Phosphoric Acid Compounds in the Blood of Normal Boys.

No.	Age.	Name.	H_3PO_4 per 100 cc												Calcium per 100 cc			CO₂	Red blood cells.	Time since last meal
			Whole blood.				Corpuscles.				Plasma									
			Total	Li-poid	Inor-ganic.	Other forms	Total	Li-poid	Inor-ganic	Other forms	Total	Li-poid	Inor-ganic	Other forms	Whole blood.	Corpus-cles	Plasma			
			mg.	*mg.*	*mg*	*mg*	*mg.*	*mg*	*mg*	*mg.*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*Vol per cent*	*per cent*	*hrs.*
1	4 wks.	B. G.	118.0	41.0	11.5	65.5	258.0	72.0	9.0	177.0	33.0	20.0	13.0	0.0	9.0	9.4	9.4	51.5	38.2	11
2	2 mos.	B. B.	127.2	45.0	9.2	73.0	324.0	74.0	12.6	237.4	43.2	35.5	8.1	−0.4			11.0*	46.2	30.4	11
3	7 “	B. S.	100.0	40.0	8.4	51.6	240.0	73.0	8.1	158.9	36.0	28.5	8.4	−0.9	9.9*	14.0*	8.4	39.5	30.1	11
4	3 yrs.	B.	128.0	50.0	9.0	69.0	279.0	72.0	11.2	195.8	45.0	37.0	7.4	0.6	11.6	11.2	11.8	50.0	36.5	15
5	4 “	W. S.	110.0	31.0	10.8	68.2	234.0	36.0	9.7	188.3	42.0	30.0	11.4	0.6	5.6	5.6	5.5	48.6	36.2	15
6	5 “	E. S.	150.0	50.0	9.2	90.8	300.0	74.0	9.2	216.8	45.6	35.0	10.0	0.6	12.4	12.0	12.4	59.3	41.4	15
7	6 “	D. K.	138.0	50.0	14.2	73.8	264.0	72.0	18.9	153.1	46.2	36.0	10.8	−0.6	11.4	11.6	11.0	50.4	42.3	15
8	6 “	J. S.	172.0*	57.0*	13.0	102.0*	360.0*	90.0*	19.6	250.4*	54.0*	45.0*	9.0	0.0	10.0	12.0	8.6	51.8	40.0	15
9	6 “	V. B.	115.0	37.0	15.0*	63.0*	240.0	55.0	31.6*	153.4*	36.0	30.0	5.0*	1.0				49.5	38.9	15
10	7 “	M. B.	110.0	37.5	16.3*	56.2*	231.0	60.0	30.4*	140.6*	30.6	23.5	7.2*	−0.1			12.0	48.5	39.5	15
11	8 “	A. G.	125.0	42.4	9.1	73.5	240.0	58.0	7.8	174.2	40.2	31.0	9.2	0.0				63.8	42.3	15
12	9 “	H. B.	130.0	47.0	11.0	72.0	270.0	64.0	17.0	189.0	45.0	37.0	7.4	0.6	8.0	6.4	9.6	51.5	38.5	15
13	9 “	W. H.	138.0	50.0	14.0	74.0	252.0	70.0	14.2	167.8	48.0	35.0	14.0	−0.1				55.1	44.0	15
14	11 “	E. J.	88.0*	41.5	12.4	34.1*	150.0*	40.4	10.0	99.6*	52.8*	40.0	12.2	0.6	8.8	5.8	10.4	52.5	36.1	15
15	12 “	R. A.	114.0	50.3	14.0	49.7	240.0	84.0	20.9	135.1	43.5	33.0	10.8	−0.3	9.2	10.0	9.4		35.9	15
16	12 “	W. J.	100.0	48.6	7.0	44.4	189.0	71.0	6.5	111.5	43.8	35.0	7.4	1.4	8.4*	5.8*	11.0*	47.6	38.1	15
17	13 “	C. R.	147.0	50.5	7.3	89.2	300.0	76.0	6.5	217.5	39.0	31.0	7.9	0.1	10.1	5.2	14.4	46.6	41.5	15
Average...........			123.3	44.5	10.6	68.8	257.4	65.7	12.1	178.6	41.1	32.3	9.8		9.4	9.0	10.2	50.8	38.2	

* Not included in the average.

CORRECTION.

On page 326, Vol. XLVII, July, 1921, in the column headed "Calcium per 100 cc." 2nd line under "Plasma" the figure 11.0 is starred and should not be, while the figure 8.4 directly under it should be. Also in the 16th line the figures 8.4, 5.8, and 11.0 are starred and should not be, while the figures 10.1, 5.2, and 14.4 directly under them should be starred.

TABLE II.

The Distribution of Calcium and Phosphoric Acid Compounds in the Blood of Normal Girls.

No.	Age.	Name.	H_3PO_4 per 100 cc.												Calcium per 100 cc.			CO_2	Red blood cells.	Time since last meal.
			Whole blood				Corpuscles.				Plasma									
			Total	Lipoid.	Inorganic.	Organic	Total	Lipoid	Inorganic	Organic	Total	Lipoid	Inorganic	Organic	Whole blood.	Corpuscles	Plasma.			
			mg.	*mg*	*mg.*	*mg*	*mg.*	*mg*	*mg*	*mg.*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg.*	*mg*	*Vol. per cent*	*per cent*	*hrs.*
1	6 mos.	B. S.	100.0	32.5			234.0	48.0			33.0	24.8	8.1	0.1	9.3	5.2	11.0	47.7	33.1	11
2	3 yrs.	A. N.	136.3	40.0	5.8	90.5	330.0	72.0	5.7	252.3	31.5	26.0	6.0	−0.5	11.3			51.8	35.5	15
3	4 "	J. S.	110.0	32.4	6.7	70.9	225.0	50.0	6.6	168.4	34.5	27.0	6.7	0.8				59.5	39.5	15
4	4 "	E. B.	116.0	37.0	9.2	69.8	240.0	52.0	9.6	178.4	37.2	28.0	9.6	−0.4	10.0	10.0	10.0	49.2	38.4	15
5	4 "	M. K.	105.0	40.8	10.0	54.2	228.0	66.0	11.6	150.4	36.0	27.0	9.0	0.0	9.1	8.4	9.6	49.2	35.6	15
6	7 "	E. C.	150.0	46.0	11.6	92.4	300.0	65.0	13.0	222.0	44.0	33.0	9.5	1.5				59.3	41.2	15
7	7 "	R. M.	122.0	37.0	14.0	71.0	252.0	58.0	26.0	168.0	36.0	26.0	8.0	2.0	9.2	8.0	10.0	48.6	40.5	15
8	7 "	G. A.	144.0	43.0	11.1	89.9	294.0	68.0	9.8	216.2	37.2	25.0	12.2	0.0	7.8	5.7	9.2	48.6	41.6	15
9	7 "	L. P.	116.2	38.5	9.3	68.4	230.0	54.4	8.5	167.1	40.0	28.8	10.3	0.9				54.3	38.0	15
10	8 "	T. P.	90.0	26.0	8.1	55.9	177.0	33.0	10.8	133.2	27.1	20.0	6.3	0.8	8.0	5.2	10.0	50.5	41.1	15
11	10 "	E. G.	142.0	48.0	13.3	80.7	279.0	63.0	10.4	205.6	55.5	40.0	15.0	0.5				55.0	41.4	15
12	10 "	V. N.	132.8	46.0	7.2	79.6	270.0	61.0	5.0	204.0	42.0	35.0	9.0	−2.0	10.0	10.4	9.7	59.5	40.3	15
13	11 "	V. R.	132.0	40.5	10.0	81.5	270.0	60.0	14.2	195.8	36.0	29.0	6.6	0.4	9.8	11.0	9.0	58.1	41.3	15
14	12 "	J. N.		51.5	10.0			71.0	9.9		47.8	37.5	10.3	0.0	8.3				42.7	15
15	12 "	E. C.	122.0	35.0	7.2	79.8	258.0	55.0	9.0	194.0	27.0	21.5	6.3	−0.8	9.1	9.6	8.8	56.2	41.2	15
16	13 "	L. P.	126.0	34.0	7.5	84.5	276.0	51.5	7.2	217.3	33.0	25.0	8.1	−0.1	10.2	10.2	10.0	47.9	37.7	15
17	13 "	E. M.	95.0	25.0	7.5	62.5	219.0	40.0	6.7	172.3	24.6	17.0	8.1	−0.5	9.8	9.4	10.0	49.8	37.4	15
Average............			121.2	38.4	9.3	75.4	255.1	55.8	10.3	189.6	36.6	27.7	8.8		9.4	8.5	9.8	52.8	39.2	
General average for children............			122.2	41.3	9.9	72.3	256.2	60.6	11.1	184.5	38.7	29.9	9.3		9.4	8.7	10.0	51.8	38.7	

41.1, while in girls the range was from 27 to 47 mg., the average being 36.6, as contrasted with Bloor's values of 32 and 36.2 mg. for men and women, respectively. In boys, the lipoid phosphoric acid content of plasma ranged from 20 to 40 mg., the average being 32 or approximately 50 per cent higher than Bloor found in men. A possible explanation of the higher values in boys than in girls or adults might be found in their greater muscular activity. The difference between the values found in girls and women is not so marked. For the former, the average was 27.7 mg. with variations from 20 to 37.5, and for the latter, 24.9 mg. The inorganic phosphoric acid values of plasma were approximately the same in boys and girls, being 9.8 and 8.8 mg. per 100 cc., respectively. The variations in the boys did not equal 100 per cent and those in the girls did not exceed 150 per cent which is in striking contrast to the percentage variation of the inorganic phosphoric acid content of the corpuscles. Marriott and Howland (6) using a different method, found the inorganic phosphorus content of the serum to vary from 1 to 3.5 mg. (approximately 3.2 to 11.2 mg. H_3PO_4) per 100 cc. in normal infants, and Denis and Minot (7) using Bloor's method found a similar range in adults suffering from various diseases other than nephritis and cardiorenal conditions. It should be noted that the sum of the inorganic and lipoid phosphoric acid values in plasma is equal to the total. Bloor found a small amount of an unknown phosphoric acid compound (up to 10 per cent of the total) in plasma. This value was obtained by subtracting that of the inorganic phosphoric acid from the acid-soluble. We did not make determinations on the acid-soluble fraction, but as the sum of the inorganic and lipoid phosphoric acid values is equal to the total, within the limits of experimental error, the presence of this substance in an appreciable quantity seems doubtful. However, in certain pathological conditions which we are now investigating, we have found considerable quantities of the so called "organic" phosphorus. In many cases it is interesting to note the approximate equilibrium of the inorganic phosphoric acid between corpuscles and plasma. This is contrary to Bloor's findings in men and women which show a much higher concentration in the corpuscles than in the plasma. We are not able to explain the significance of the differences between boys and girls and between children and adults.

Until recently, it has generally been conceded that little or no calcium exists in the corpuscles. Howland and Marriott (8) found about half as much calcium in normal whole blood as in serum, from which they concluded that there was no calcium in the corpuscles and advocated the use of serum or plasma for calcium determinations. Many references to their work have been found in the literature. Hammarsten (9) cites the work of Gryns, Köppe, Hamburger, and others showing that blood cells are impermeable to calcium and magnesium, although Schmidt (10) in 1850 claimed that corpuscles contain a considerable amount of this metal. Recent investigations of Cowie and Calhoun (11) and others have confirmed Schmidt's contention by showing that calcium is present in corpuscles in appreciable quantities. Cowie and Calhoun used Lyman's method and made many determinations on a few cases, the values in one man being as follows: whole blood, 8.9 mg.; corpuscles, 4.26 mg.; and plasma, 12.07 mg. per 100 cc. We were not able to ascertain whether the determinations were made on the same or different samples of blood. Brown, MacLachlan, and Simpson (12) also using Lyman's method found an average of 9.5 mg. of calcium per 100 cc. of whole blood in eighteen normal infants, which is in close agreement with our value of 9.4 mg. Lyman reported an average of 6.1 mg. per 100 cc. of whole blood for men and 7.1 mg. for women. One woman had a value of 9.4 mg. The great majority of investigators have used serum or plasma for their determinations, the reported values of 9 to 11 mg. agreeing well with our average of 10 mg. per 100 cc. of plasma. The most striking feature of this investigation is the large amount of calcium found in the corpuscles, in many cases the content being equal to or greater than that in the plasma. As was found in the case of inorganic phosphoric acid, the greatest percentage variation of the calcium content occurred in the corpuscles, the boys showing wider limits than the girls. One boy, age 4 years, had an extremely low concentration in the blood, his values being: whole blood, 5.6 mg.; corpuscles, 5.6 mg.; and plasma, 5.5 mg. per 100 cc. His phosphoric acid values were well within the normal range. Neither age (within the above range) nor sex appears to be a factor in the calcium concentration of the blood or its distribution between plasma and corpuscles. Meigs, Blatherwick, and Cary (13) showed that in heifers the calcium content of plasma tends to become lower with advancing age up to 6 months. In some studies on the new-born now in progress, we have found the average calcium values of five babies under 14 hours of age to be as follows: whole blood, 8.7 mg.; corpuscles, 5.9 mg.; and plasma, 12.6 mg. per 100 cc. While we have not yet sufficient data from which we can draw conclusions, it appears that the calcium content of plasma is higher at birth than in later life, the average value for corpuscles being markedly less than that in older children.

The plasma of thirty-two children had an average CO_2-combining power of 51.8 volumes per cent. Sawyer, Stevens, and Bauman (14) report a plasma carbonate value of 54 volumes per cent in children between the ages of 4 and 8 years. Schloss and Stetson (15) found a range of from 46.1 to 76.1 volumes per cent in normal infants. Only one of our values (39.5) was less than Schloss and Stetson's lower limit, and we consider this too low to be within the normal range. It is interesting to note that in this case the calcium content of plasma is lower than the average and that of the corpuscles is considerably higher.

SUMMARY.

The alkali reserve of plasma and the distribution of calcium and the various compounds of phosphoric acid in the blood were studied in thirty-four normal children whose ages ranged from 4 weeks to 14 years.

From the above data it appears that the blood corpuscles are richer in all types of phosphoric acid compounds than plasma. The amount of unknown phosphoric acid in plasma is negligible, if any, while in corpuscles it averages approximately 70 per cent of the total.

In general, the values for boys averaged slightly higher than those for girls. The lipoid phosphoric acid content of corpuscles averaged 17.7 per cent higher in boys than in girls, while the plasma value in boys was 16.6 per cent higher than that in girls. The inorganic phosphoric acid content of corpuscles showed the greatest percentage variation of all the phosphorus compounds of the blood.

The average calcium content of corpuscles was found to be slightly less than that of the plasma, the values in mg. per 100 cc. being as follows: whole blood, 9.4 mg.; corpuscles, 8.7 mg.; plasma, 10.0 mg. A relation between the calcium and phosphoric acid contents of the blood is not apparent.

The CO_2-combining power of the plasma averaged 51.8 volumes per cent in thirty-two children. No relation between the alkali reserve and the concentration of calcium and phosphoric acid in the blood can be established.

BIBLIOGRAPHY.

1. Sundstroem, E. S., and Bloor, W. R , *J. Biol. Chem.*, 1920-21, xlv, 153.
2. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.
3. Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.
4. Van Slyke, D. D , and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
5. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 49.
6. Marriott, W. McK., and Howland, J., *Arch. Int. Med.*, 1916, xviii, 708.
7. Denis, W., and Minot, A. S , *Arch Int. Med* , 1920, xxvi, 99.
8. Howland, J., and Marriott, W. McK., *Quart J. Med.*, 1917-18, xi, 289.
9. Hammarsten, Text-book of physiological chemistry, New York, 1908, 195.
10. Schmidt, C., quoted in Mathews, A. P., Physiological chemistry, New York, 1915, 462.
11. Cowie, D. M., and Calhoun, H. A., *J. Biol. Chem.*, 1919, xxxvii, 505.
12. Brown, A., McLachlan, I. F., and Simpson, R., *Am. J. Dis. Child.*, 1920, xix, 413.
13. Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.
14. Sawyer, M., Stevens, F. A., and Bauman, L., *Am. J. Dis. Child.*, 1918, xv, 1.
15. Schloss, O. M., and Stetson, R. E., *Am. J. Dis. Child.*, 1917, xiii, 218.

STUDIES OF AUTOLYSIS.

VII. AUTOLYSIS OF BRAIN.

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Brain undergoes autolytic decomposition very slightly when measured by amino-acid production and when compared with most other tissues. The evidence of the reaction even under the most favorable conditions is so slight that it may easily be overlooked, or be incorrectly interpreted. It is because of this that the brain proteins are assumed (1) to be peculiarly resistant to autolytic changes and not subject to those rather rapid fluctuations of mass which occur for example in the liver, and in other organs. The peculiar stability of brain and nerve proteins thus appears to relate itself to those functions of the brain which seem to demand a peculiarly stable physical structure, the functions of memory, habit, instinct, etc.

While there can be no question about the stability of the protein framework of the brain cells, it does not necessarily follow that the autolytic mechanism is wanting, or is strikingly different from that of other organs. The outstanding fact of stability and permanence of brain cell structure may equally well be explained by the peculiarly perfect protection against local acidosis which the large blood supply of the brain insures. The respiratory center is itself nerve tissue. It is characterized by its sensitiveness to H ion changes, to CO_2 and O content (2-8). By its regulation the blood always supplies the brain with oxygen sufficient to prevent asphyxial reactions from developing, except under pathological conditions. With abundance of oxygen supplied, there is also very perfect CO_2 elimination, and a supply of buffer salts which tends to prevent the development of local acidosis. In short, the regulation of CO_2 elimination, of constant H ion level, and of oxygen supplied the body is very largely effected by a portion of the central nervous system itself. Conditions giving rise to acidosis of the liver, a group of muscles, or the kidney may be without influence on the brain, or on tissue respiration as a whole. On the other hand any condition of the brain tending toward acidosis, asphyxia, faulty CO_2 elimination, or rising H ion concentration, will result in an immediate respiratory and circulatory response affecting the entire body. The brain may be said to protect itself from asphyxia and acidosis, and incidentally in so doing to protect the rest of the body. If the brain tissue is like other tissues it will not exhibit autolytic changes as long as it is so perfectly protected against the conditions under which alone autolysis can go on.

Clinically we have much evidence to support the idea that atrophic changes do go on in the brain, just as they do in other tissues. We have the familiar losses of consciousness in brain anemias or asphyxias, rapidly progressing to more permanent functional losses and death where the condition is at all prolonged. General acidosis frequently leads to coma from which no measure of relief can save the patient, suggesting a progressive degenerative change in the central nervous system of an irreversible character. Local pressures in the brain lead to paralyses, and these may become permanent as a result of irreversible atrophic changes. The pathologist recognizes and correlates functional losses with brain or nerve degeneration. These degenerations are perfectly analogous to the atrophies of other tissues. They suggest that under favorable conditions autolysis may proceed quite rapidly, and in the delicate and essential protein structures of the brain cells produce irreversible losses and obliterations of a framework upon which the unique characteristics of the central nervous tissue depend.

It appeared worth while, therefore, to review and repeat such significant work as has been reported on brain autolysis and to compare the autolytic reaction of brain with that of other tissues. Levene and Stookey (9) showed in 1903 that brain autolysis produces such characteristic protein decomposition products as peptones, amino compounds, and free NH_3, while the coagulable protein fraction diminishes. They found that acids accelerate this process, while alkalies inhibit it. Their experiments were few, however, and confined to the demonstration of the fact of brain autolysis, which had not previously been convincingly shown.

EXPERIMENTAL PART.

The experimental technique is exactly like that described in previous papers (10). 50 gm. of brain tissue, finely ground, are made up to 250 cc. with water and toluene. 25 cc. samples are taken, made up to 100 cc. with trichloroacetic acid to precipitate the proteins and 25 cc. aliquots of the filtrates titrated by the formol method for amino-acids The titration figures are small and the errors due to end-points are proportionately large. By matching end-point colors with a standard and by using small burettes graduated in 0.01 cc., the titration errors have been kept as low as possible and the figures, though actually small, are nevertheless characteristic and significant.

A few typical examples, selected from the large number of experiments performed, are presented.

From Tables I to V and Fig. 1 we are able to conclude that brain pulp autolyzes normally as do other tissues *in vitro*. The

TABLE I

Sheep Brain.

No.	Condition	0.20 N amino-acids					Net gain in 33 days
		Days					
		0	2	3	10	33	
		cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
I	Control	0.40	0.55	0.65	0.85	1.25	0.85
II	" in 0.01 N HCl	0.40	0.55	0.68	1.00	1.50	1.10
III	" " 0.02 N HCl.....	0.40	0.57	0.65	1.15	1.45	1.05
IV	" " 0.04 N HCl... ...	0.40	0.50	0.65	0.70	0.90	0.50
V	" + 3 gm. Na_2HPO_4	0.40	0.55	0.60	0.80	1.05	0.65

TABLE II.

Calf Brain.

No.	Condition.	0.20 N amino-acids				Net gain in 30 days.
		Days				
		0	1	5	30	
		cc.	*cc.*	*cc.*	*cc.*	*cc.*
I	Control..............................	0.25	0.35	0.60	0.60	0.35
II	" in 0.01 N HCl................	0.25	0.40	0.90	1.40	1.15
III	" " 0.02 N HCl................	0.25	0.40	0.80	1.30	1.05
IV	" " 0.01 N NaOH..............	0.25	0.25	0.35	0.45	0.20
V	" + 5 gm. $CaCO_3$..............	0.25	0.40	0.45	0.60	0.35

TABLE III.

Calves Brains.

No.	Condition.	0.20 N amino-acids.							Net gain in 30 days.	
		Days.								
		0	1	2	5	10	20	30	10	30
		cc	cc	cc	cc	cc	cc	cc.	cc.	cc.
I	Control	0.3	0.35	0.37	0.40	0.45	0.50	0.55	0.20	0.25
II	" in 0.005 N HCl.	0.3	0.4	0.55	0.65	0.80	0.90	1.00	0.60	0.70
III	" " 0.01 N HCl.	0.3	0.4	0.60	0.65	1.00	1.05	1.40	0.80	1.10
IV	" " 0.02 N HCl.	0.3	0.4	0.70	0.75	1.00	1.40	1.60	1.10	1.30
V	" " 0.04 N HCl..	0.3	0.25	0.30	0.33	0.40	0.40	0.40	0.10	0.10
VI	" " 0.1 N HCl..	0.3	0.25	0.30	0.30	0.35	0.45	0.55	0.15	0.25
VII	" " $NaHCO_3$.. ..	0.3	0.35	0.35	0.35	0.40	0.45	0.50	0.15	0.20
VIII	" " K_2HPO_4......	0.3	0.35	0.35	0.35	0.40	0.40	0.50	0.10	0.20

TABLE IV.

Calves Brains.

No	Condition	0.20 N amino-acids.				Net gain in 15 days
		Days.				
		0	1	3	15	
		cc.	cc	cc	cc.	cc.
I	Control..........................	0.25	0.40	0.50	0.50	0.25
II	" in 0.04 N HCl.............	0.25	0.60	0.55	0.60	0.45
III	" " 0.02 N HCl........	0.25	0.50	0.70	0.80	0.65
IV	" + 1 gm. K_2HPO_4....... .. .	0.25	0.40	0.50	0.45	0.20
V	" + 2 " K_2HPO_4.	0.25	0.40	0.40	0.40	0.15
VI	" + 5 " K_2HPO_4.............	0.25	0.35	0.30	0.30	0.05
VII	" + 10 " K_2HPO_4.............	0.25	0.40	0.30	0.35	0.10

TABLE V.

Sheep Brain, Foreign Protein, and Acid.

No.	Condition.	0.20 N amino-acids.					Net gain.	Digestion of foreign protein.
		Days.						
		0	1	3	7	15		
		cc.	cc.	cc.	cc.	cc	cc.	
I	Control.,................	0.25	0.50	0.50	0.60	0.60	0.35	
II	" in 0.04 N acid....	0.25	0.60	0.65	0.65	0.70	0.45	
III	" " peptone.......	0.75	1.90	2.10	2.30	2.40	1.65	1.25
IV	" " " acid..	0.75	1.40	1.30	1.50	1.70	0.95	0.50
V	" " gelatin......	0.40	0.70	0.90	1.00	1.10	0.70	0.25
VI	" " " acid..	0.40	1.20	1.50	1.60	1.90	1.50	1.05

amount of protein in brain is small, approximately 7 per cent of the total, while in muscle it approximates 18 to 20 per cent, and in liver 15 per cent or more. It is not surprising, therefore, that the figures for brain autolysis should be much smaller than those for liver, muscle, and other tissues. The addition of acid increases the speed and extent of autolysis of brain, but the amount required for maximum digestion is smaller than for tissues like the liver.

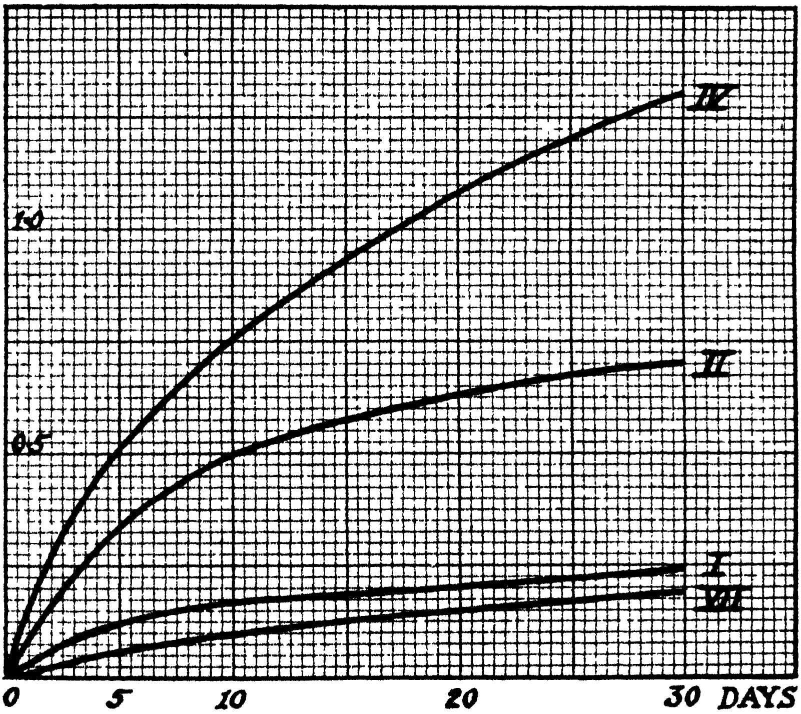

FIG. 1.

This is correlated evidently with the protein content, the more protein present the more acid can be added before the H ion rises to the point of inhibiting the reaction. With liver, sufficient acid to make the digesting mixture 0.04 to 0.1 N gives maximum autolysis. With brain an acidity of 0.02 N is optimum.

Foreign proteins such as gelatin and peptone, which are digested by the autolytic enzymes of the liver, are also found to digest in the brain mixtures. It is of interest to note that peptone digests better in the nearly neutral control than it does in the rather strongly acid brei used, while gelatin digests much better in the acid medium.

The stability of brain structure depends not on any lack of proteolytic enzymes in the cells, but upon the fact that normally the brain proteins are not available substrata for the enzymes. They become available under the conditions that make liver proteins available for autolytic hydrolysis; namely, acidosis within the cells. That local acidoses do not frequently occur in brain tissue in spite of its large CO_2 production, is due we believe to the exceptionally large blood supply to that organ, and to its ability to modify the respiratory and circulatory rate so as to prevent any accumulation of CO_2 or other acid. By its extreme sensitiveness to increased H ion concentration and CO_2 and by its position as master tissue of the body it automatically prevents just those conditions from arising within itself which would eventuate in its own autolytic disintegration.

SUMMARY.

1. Brain tissue autolyzes in the same way as other tissues thus far examined though quantitatively on a smaller scale comparable to the low protein content of brain tissue.

2. The speed and extent of the proteolysis is determined by the H ion concentration of the mixture. In alkaline or neutral media autolysis is inhibited. It is increased in proportion to the acid added.

3. The optimum acidity for autolysis of brain tissue is about 0.02 N, or much less than that for liver, kidney, and other epithelial tissues. The small amount of acid required corresponds to the small amount of protein present and made available for digestion by the addition of acid.

4. Brain cells contain proteolytic enzymes which digest such added substrata as gelatin or peptone, as in other gland structures.

5. The permanence of the protein structures of the brain appears to depend on the very perfect protection against asphyxia and CO_2 accumulation, which its large blood supply and its control of respiration insures. When asphyxia and acidosis do develop in brain tissue, it autolyzes like other tissues.

6. The autolytic disintegration of the delicate protein structures of brain tissue appears to be an irreversible phenomenon, and is accompanied by loss of such characteristic functions as memory, habit, motor control, and consciousness.

BIBLIOGRAPHY.

1. Mathews, A. P., Text-book of physiological chemistry, New York, 3rd edition, 1920, 590.
2. Gasser, H. S., and Loevenhart, A. S., *J. Pharmacol. and Exp. Therap.*, 1913 14, v, 239.
3. Haldane, J. S., and Priestly, J. G , *J. Physiol.*, 1905, xxxii, 225.
4. Hasselbach, K. A., *Biochem. Z.*, 1912, xlvi, 403.
5. Winterstein, H., *Arch. ges. Physiol.*, 1911, cxxxviii, 167.
6. Campbell, J. M. H., Douglas, C. G., Haldane, J. S., and Hobson, F. G., *J. Physiol.*, 1913, xlvi, 301.
7. Douglas, C. G., *Ergebn. Physiol.*, 1914, xiv, 338
8. Stewart, G. N., Guthrie, C. C., Burns, R. L , and Pike, F. H., *J. Exp. Med* , 1906, viii, 289
9. Levene, P. A , and Stookey, L. B., *J. Med. Research*, 1903-04, x, 217.
10. Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

A STUDY OF THE CATALASE REACTION.

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The significance of catalase in the living organism is still an open question. Only one fact has thus far been incontestably established; namely, that catalase liberates molecular oxygen from hydrogen peroxide, though it fails to do so with similarly constituted organic compounds like ethyl hydrogen peroxide.

In a sense catalase lends itself admirably to the investigation of the dynamics of its action. The course of the reaction can be easily and directly timed and measured by the evolution of oxygen. Furthermore, the interference generally caused through an accumulation of products of the reaction is absent here since, except for very minute traces of oxygen remaining in solution, the gaseous by-product is quickly driven off, especially when the mixture is vigorously shaken.

I.

All the experiments reported in this paper were performed with a crude catalase preparation from liver.[1] The finely ground pulp of beef liver was extracted with chloroform-water over night and the liquid was strained through cloth to remove solid particles. Enough alcohol was then added to this liquid to make a 50 per cent mixture,·when a massy precipitate forms on standing. This precipitated material is collected on a hardened filter paper and the excess fluid removed by suction. The material is then dried in the air and ground to a fine powder in a mortar. The powder possesses strong catalase action. During several months the reactivity of the preparation has apparently remained unchanged. A more highly purified sample of catalase can be prepared from this powder by reprecipitating a watery extract with an equal volume of alcohol. Since repeated precipitation causes great loss of the catalase, the crude preparation was used exclusively in these experiments. A definite quantity of this dry powder was weighed out (usually 0.5 gm.) and extracted with 200 cc. of chloroform-water. A water-clear extract is obtained which, when kept in an ice chest, retains its strength undiminished for a considerable time. The extract was neutralized with 0.01 N NaOH to make its pH = 7.0. The hydrogen ion concentration in all these experiments has been determined colorimetrically with the aid of the Hynson, Westcott and Dunning standards. The relative amount of catalase was measured by the number of cc. of the extract employed.

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[1] In some experiments with kidney extracts which were preliminary to this research the author enjoined valuable aid from Dr. V. E. Levine (*Science*, lii, 612).

The hydrogen peroxide used in the experiments was the grade known under the trade name of "Oakland Dioxogen." Inasmuch as the hydrogen peroxide is strongly acid it was first neutralized with NaOH and its hydrogen ion concentration adjusted to a pH somewhat below 7.0. The reason for this procedure is that the hydrogen peroxide decomposes spontaneously when it is neutral or alkaline in reaction. On this account also just enough peroxide was prepared for the day's work; a fresh supply being made for each occasion. The strength of the hydrogen peroxide was standardized by titrating against 0.1 N potassium permanganate, and expressed in terms of its oxygen content. When a series of experiments lasted several hours the titer of the hydrogen peroxide was again checked up at the end of the experiment.

II.

The apparatus in which the catalase activity was measured (Fig. 1) consisted of three parts: (1) a shaker driven by an electric motor; (2) an Erlenmeyer flask in which the reaction between the catalase and the hydrogen peroxide took place; and (3) a eudiometer tube in which the gas was collected for measurement. The volume of gas was reduced to standard conditions of temperature and pressure, and all the data recorded in the paper represent the corrected volume. The catalase preparation was measured into the Erlenmeyer flask and enough water added to secure the desired relative concentration. In some experiments the same volume was maintained throughout the entire series. The hydrogen peroxide was measured into a glass cup provided with a syphon. The cup was suspended from the bottom of a rubber stopper with which the Erlenmeyer flask was closed. The stopper bore a plunger which could be easily pushed down into the cup by pressing on the rod extending to the outside. The descent of the plunger raised the level of the liquid in the cup and started the syphon which drained the contents in about 10 seconds. As soon as the cup was emptied the shaking was

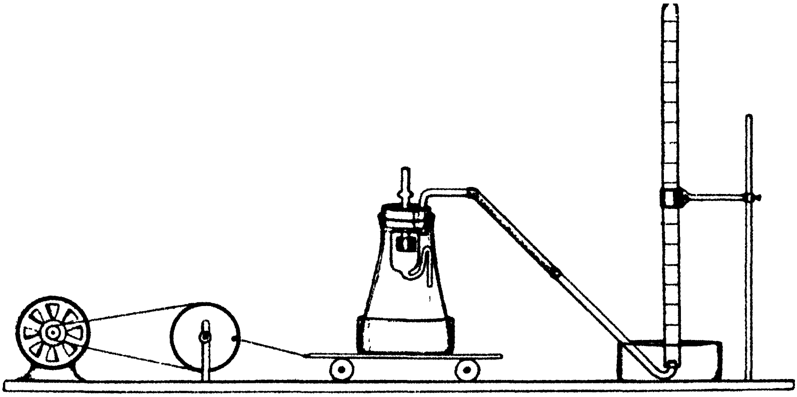

Fig. 1.

started by turning the motor switch. The evolution of the gas was recorded at the end of every 5 minutes, and the shaking continued until no more gas was given off. Usually experiments required about one-half to three-quarters of an hour for completion.

III.

To insure its stability the commercial hydrogen peroxide is strongly acidified. When this acid hydrogen peroxide is used a serious error is introduced into the experiments, which seems to have been the case in the older experiments on catalase. Even when hydrogen peroxide is made neutral to Congo red, the peroxide is still strongly acid as far as its hydrogen ion concentration is concerned. Certainly, in a series of experiments where the quantity of hydrogen peroxide is varied, the range of variation in hydrogen ion concentration may be very great. To avoid such a possibility, all our experiments were conducted at a definite hydrogen ion concentration. This end was accomplished either with the aid of appropriate phosphate buffer mixtures, or by bringing both the hydrogen peroxide and the catalase solution to a definite pH by adding 0.01 N NaOH. The objection to the use of the buffer is that, especially at a pH over 7.0, there is danger of the hydrogen peroxide being broken up by it. While within the short time of the catalase experiment this danger is greatly minimized, nevertheless, the second method was used in preference except where a wide range of pH values was required.

The first thing, of course, was to determine the optimum hydrogen ion concentration for the catalase reaction. Experiments were performed in which the only variable factor was the pH of the mixture, adjusted by means of phosphate buffers. The pH of the mixture was invariably checked up at the close of each experiment.

The series reported below has been made with 5 cc. of the catalase extract and hydrogen peroxide sufficient to yield 173 cc. of oxygen (determined by titration); 20 cc. of the appropriate buffer mixture were used in each experiment. The initial hydrogen peroxide concentration was 0.31 gram-molecular in all these experiments. For reasons which will become apparent from subsequent discussion the quantity of hydrogen peroxide and catalase was so adjusted that the curve of the oxygen evolution followed very closely the isotherm of a bimolecular reaction

$$K = \frac{1}{at} \cdot \frac{x}{a-x}$$

It is obvious from these experiments that the optimum condition for the catalase reaction is at neutrality (pH = 7.0). As the acidity of the medium increases, both the velocity of the reaction and the total amount of peroxide decomposed diminish somewhat at first, but when the pH is below 6.0 the decrease becomes very noticeable. Thus, at pH = 6.4 the reaction is 98 per cent complete, but at pH = 5.8 it is 88 per cent, and at pH = 5.2 it is only 70 per cent of that at the pH = 7.0. Increasing the hydroxyl concentration (pH = 7.2 to 8.3) has apparently little or no effect on the amount of hydrogen peroxide decomposed, though the reaction velocity is somewhat diminished.

TABLE I.

pH	Time.	Oxygen evolved.		$K \times 10^5$	H_2O_2 decomposed.
		Observed	Calculated.		
	min.	*cc.*	*cc.*		*per cent*
	5	50.7	44.5	48	
	10	75.1	70.8	44	
5.2	15	88.9	88.1	41	55
	20	94.0	100.4	34	
	23	95.3	109.2	31	
				—	
				40	
	5	62.5	56.5	66	
	10	90.2	85.2	63	
	15	105.2	102.5	60	
5.8	20	113.3	114.1	55	69
	25	117.1	122.5	48	
	31	119.7	129.8	42	
				—	
				56	
	5	62.9	63.5	66	
	10	93.6	92.9	68	
	15	113.4	109.8	73	
6.4	20	124.2	120.8	74	76
	25	128.3	128.6	66	
	29	131.2	134.3	63	
	33	132.7	137.0	58	
				—	
				67	
	5	65.1	65.8	70	
	10	95.3	95.3	71	
	15	114.3	112.4	75	
7.0	20	125.2	122.8	75	79
	25	130.9	130.4	72	
	29	133.9	135.9	69	
	34	136.1	140.2	63	
				—	
				71	

TABLE I—*Concluded.*

pH	Time	Oxygen evolved.		$K \times 10^5$	H_2O_2 decomposed.
		Observed	Calculated.		
	min.	*cc.*	*cc.*	*per cent*	*per cent*
7.2	5	60.3	59.7	62	75
	10	89.9	88.8	63	
	15	108.2	106.1	64	
	20	119.4	117.4	64	
	25	126.2	125.4	62	
	31	130.0	131.2	56	
				—	
				62	
7.5	5	60.1	60.5	62	78
	10	88.2	89.6	60	
	15	108.0	106.9	64	
	20	120.6	118.2	67	
	25	128.3	126.3	62	
	30	132.8	132.2	64	
	35	135.2	136.9	59	
				—	
				62	
8.3	5	59.1	60.5	60	79
	10	86.4	89.6	58	
	15	107.8	106.9	65	
	20	120.0	118.2	65	
	25	127.6	126.3	65	
	30	133.0	132.2	64	
	35	136.1	136.9	60	
				—	
				62	

The fact of particular significance, however, is that while the reaction runs true to the course of a bimolecular curve within the range of hydrogen ion concentrations of from 6.4 to 8.3 (and about three-quarters of the peroxide undergoes decomposition), the reaction deviates from this course as the acidity exceeds pH = 6.4.

Although pH = 7 has been found to be the optimum hydrogen ion concentration for the catalase reaction, the experiments were made in a medium slightly on the acid side (pH = 6.7 to 6.9) in order to guard against spontaneous decomposition of the hydrogen peroxide which happens most readily at pH = 7.0 and above.

IV.

An examination of the effect of changing the quantity or the concentration of the hydrogen peroxide on the catalase reaction brings to light a number of interesting points. When a series of experiments is performed with a constant quantity of catalase it becomes at once apparent that, as the hydrogen peroxide increases, the reaction proceeds more slowly. Furthermore, except where there is a considerable excess of catalase, only a certain and variable portion of the peroxide will be decomposed. Indeed, with every increase in the amount of hydrogen peroxide there is a diminution in the proportion which is decomposed with the liberation of its oxygen. This will be seen from a series of experiments with a constant amount of catalase and a variable amount of hydrogen peroxide, recorded graphically in Fig. 2.

These experiments were made with 5 cc. of the catalase preparation. The total volume of the mixture was 50 cc., and the reaction of the mixture was maintained at pH = 6.9 throughout the series. The temperature in this as well as all other experiments was 20–21°C. In this series the hydrogen peroxide used represented a range of variation of from 45 to 226 cc. of available oxygen, as determined by titration with permanganate. Since the final volume was the same in all experiments, the concentration of the peroxide varied from 0.08 to 0.40 gram-molecular. The smallest concentration was so much below the catalase capacity that it has been left out of the graphic record. With a gram-molecular concentration of 0.16 (*i.e.*, with 90 cc. of oxygen available) 94.2 per cent of the hydrogen peroxide was decomposed in 12 minutes when the reaction came to an end. With an increase in the concentration up to 0.32 gram-molecular the amount of oxygen evolved gradually rises but the proportion of hydrogen peroxide decomposed diminishes. Thus, while the amount of oxygen liberated increases from 85 to 122.7 cc. the percentage of hydrogen peroxide decomposed diminishes from 94.2 to 68.2 per cent. When the concentration of the hydrogen peroxide is greater than 0.32 gram-molecular, the quantity of oxygen actually set free in the reaction begins to decline. At concentrations below 0.16 gram-molecular the catalase is so much

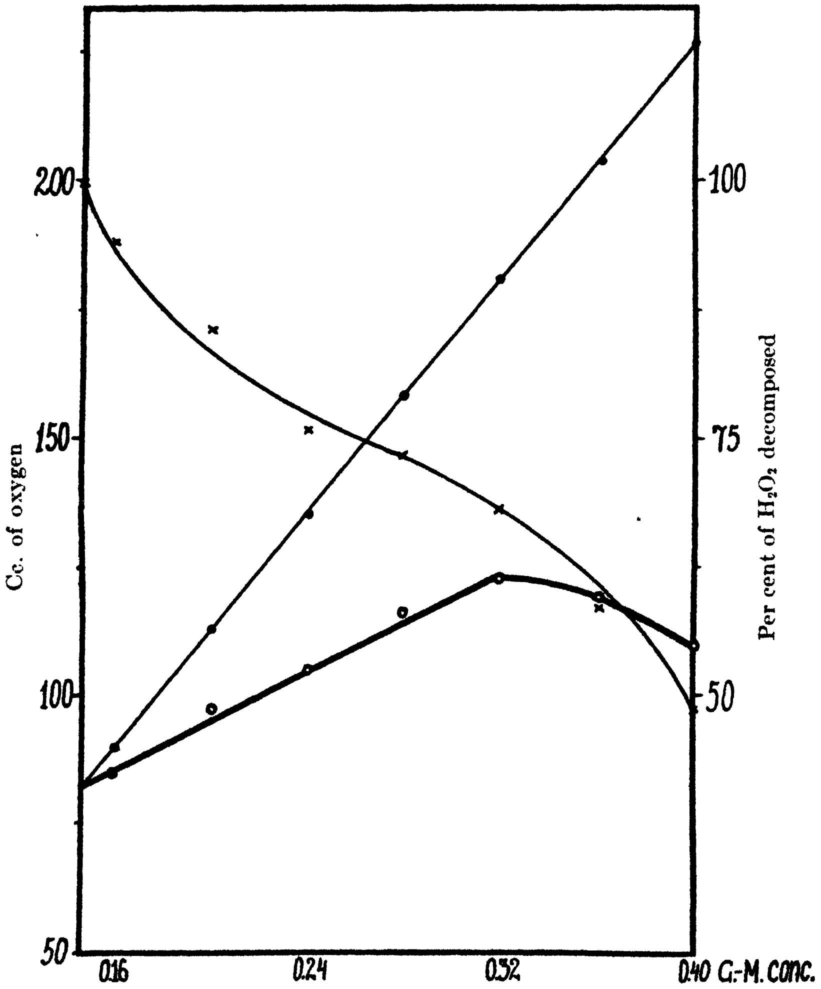

FIG. 2. Effect of increasing quantities of hydrogen peroxide on the catalase reaction. •—• Cc. of oxygen available. ○—○ Cc. of oxygen liberated. ×—× Per cent of hydrogen peroxide decomposed.

in excess that the hydrogen peroxide is completely decomposed. Considering the regularity of the curve, it is reasonable to assume that with hydrogen peroxide equivalent to 85 cc. of oxygen the decomposition would be 100 per cent complete.

The decline in the absolute quantity of oxygen liberated with the same amount of catalase requires further consideration. This may have been either due to an increase in the amount of hydrogen peroxide or to its greater concentration. It might be supposed, for instance, that the peroxide contains some impurity which is too negligible when the quantity of peroxide is small, but when more of this is used there is also enough of the injurious substance to retard and depress the reaction. Such a view, however, is untenable because it can be shown that the depressing effect may be produced by the same quantity of hydrogen peroxide when its concentration is increased. Thus in an experiment with the same amounts of peroxide and of catalase the volume was so adjusted that in one case the peroxide was in a concentration of 0.18 and in the other of 0.36 gram-molecular. In the former case the reaction ended with the production of 87.3 cc. of oxygen, while in the latter, only 78.2 cc. were set free. On the other hand, it must be emphasized that the change in concentration of the peroxide is not the only and effective factor in depressing the reaction. Even with a constant concentration of peroxide, as soon as the hydrogen peroxide quantity has been increased beyond a certain point, the reaction is progressively depressed.

V.

The depressing influence exercised by hydrogen peroxide on the catalase reaction has been invariably observed also by the older investigators of the subject. It has been generally noted that the amount of oxygen evolved diminishes, but the effect was attributed by them to a destruction of the catalase through oxidation by the excess of hydrogen peroxide. Indeed, the fear of oxidizing the catalase with the hydrogen peroxide was so predominant in the earlier work on catalase that the reaction was carried out at very low temperatures (0°C.) and with very dilute hydrogen peroxide solutions. This has occasioned certain errors in interpretation to which we shall return later. In the older experiments on catalase no heed was paid to the hydrogen ion concentration of the reaction medium. Since peroxide is usually strongly acid, and the relative acidity would increase with the increase in the quantity of the hydrogen peroxide employed, it is not improbable that the very great depression observed under these conditions was primarily the effect of greater acidity. The considerable depression (a loss of 30 per cent in catalase activity) brought on by a change in the reaction of the medium only from pH 7 to 5.2 has already been demonstrated in the foregoing.

Experiments in which the depressing effect was produced by merely increasing the concentration of the peroxide without actually changing its quantity convinced us that the apprehension that the catalase may be oxidized by the peroxide is entirely unfounded. But to test this matter further a series of experiments was performed in which instead of a small excess of hydrogen peroxide *very large* excesses were used. These experiments prove definitely the inadequacy of the idea of the destruction of the catalase through oxidation with hydrogen peroxide. In the experiments reported below hydrogen peroxide of such strength was used that it bleached and even blistered the skin of the hands. One could naturally expect that a few milligrams of catalase would be quickly and completely destroyed by so powerful an oxidizing reagent. This, however, was not the case, and the reaction, though much retarded and suppressed, continued for a considerable length of time, as can be seen from the data in Table II.

TABLE II.

Hydrogen peroxide		Oxygen evolved in minutes				H_2O_2 decomposed
Concentration	Oxygen	10	20	30	40	
mol	*cc*	*cc*	*cc*	*cc*	*cc*	*per cent*
0.2	112	53.5	75.8	85.8	91.2	82.1
0.4	224	49.6	60.7	74.9	76.6	34.2
0.8	448	30.3	43.7	53.2	57.5	13.6
1.6	896	23.0	29.9	30.3	34.1	3.8
2.4	1,344	19.5	23.0	23.7		1.8

It is obvious from these results, therefore, that the diminished catalase activity which occurs when the hydrogen peroxide is increased beyond a certain optimum quantity can be accounted for neither on the assumption that it is due to some incidental impurity, nor that the catalase undergoes destructive oxidation. Two things, however, can be regarded as proven: First, that when the quantity of hydrogen peroxide is above a certain minimum, which the catalase can break up completely, the amount of oxygen evolved gradually rises to a maximum point with an increase in the amount of hydrogen peroxide. In a number of experiments it has been established that with our liver catalase preparation the maximum oxygen evolution occurs when 65 to 70 per cent of the total hydrogen peroxide is decomposed. Secondly, when the maximum evolution of oxygen has been reached, further increases in the quantity of the hydrogen peroxide, even when not considerable, cause a falling off in the amount of oxygen set free in the reaction. This fact permits apparently of only one interpretation; namely, that we are dealing with a reversible reaction. This view will receive further support when we consider the nature of the catalase reaction.

VI.

The question naturally presented itself whether or not all the catalase is used up in the reaction when 65 to 70 per cent of the hydrogen peroxide breaks up setting free its oxygen. The following experiments were made with this in view. 5 cc. of the liver catalase solution were allowed to react with a quantity of hydrogen peroxide equivalent to 173 cc. of oxygen. In 30 minutes the reaction was complete when 120.1 cc. of oxygen were set free. In other words, 69 per cent of the hydrogen peroxide was decomposed. If free catalase were still present in the mixture it would be impossible to explain why the remaining 52.9 cc. failed to be liberated. Indeed, when another 5 cc. of catalase are now added to the system from which no more oxygen came off, the previously undecomposed quantity breaks up very rapidly. In 8 minutes 52.1 cc. of oxygen are given off which is practically the theoretically expected amount. We must, therefore, conclude that when the reaction came to a stop with the evolution of 120.1 cc. of oxygen that there was no more active catalase present in the system. The experiment, under precisely the same conditions, was then repeated with a certain modification. If a certain amount of catalase can decompose only a definite quantity of hydrogen peroxide and is thereby itself used up, will this happen also when the reaction occurs in more than a single stage?

5 cc. of the catalase preparation were again allowed to react but this time with only about half the quantity of hydrogen peroxide (92 cc. of O_2). The reaction went off much quicker than before, and in 10 minutes it was all over while practically the entire theoretical amount of oxygen was set free (91.7 cc.). More hydrogen peroxide was then added to bring the total amount of available oxygen to 180 cc. of oxygen. Upon the addition of the second quantity of hydrogen peroxide the reaction started up once more, this time, however, much more slowly, and in 15 minutes 26.4 cc. were liberated. Altogether, therefore, 118.1 cc. of oxygen were given off in the two consecutive stages, which compares very favorably with the former result, when the entire peroxide quantity reacted at once with the catalase. The somewhat smaller amount of gas formed may perhaps be due to the fact that in the second experiment there was about 5 per cent more peroxide than before. The experiment was then tried with still another variation. Starting with a quantity of hydrogen peroxide equivalent to 180 cc. of oxygen it was made to react with only 3 cc. of the catalase preparation. The reaction ran to completion in 29 minutes with the liberation of 71.2 cc. of oxygen. There was still, therefore, an excess of 108.8 cc. present. When now 2 more cc. of the catalase were added to the mixture, the reaction commenced again but at a very slow rate and ceased in 25 minutes when 52.3 cc. of oxygen were liberated. Thus, 123.5 cc. of oxygen were produced, or 69 per cent of the peroxide decomposed, when 5 cc. of catalase were used though the reaction occurred now in two stages. The conclusion seems, therefore, justifiable that the catalase is used up in the reaction, and that it reacts with a definite quantity of hydrogen peroxide. The reaction is essentially the same as that between an acid and a base, or perhaps more correctly as between an alcohol and a fatty acid. It likewise seems highly probable from our evidence that when the hydrogen peroxide is 65 to 70 per cent decomposed the liver catalase is completely used up.

VII.

The study of the relation between varying amounts of catalase and the quantity of oxygen liberated from a definite amount of hydrogen peroxide gains particular interest in the light of the experiments just discussed. In the preceding series of experiments using a constant catalase quantity and different amounts of hydrogen peroxide it was found that, at any rate within a limited range of variations of the peroxide, there is a regular

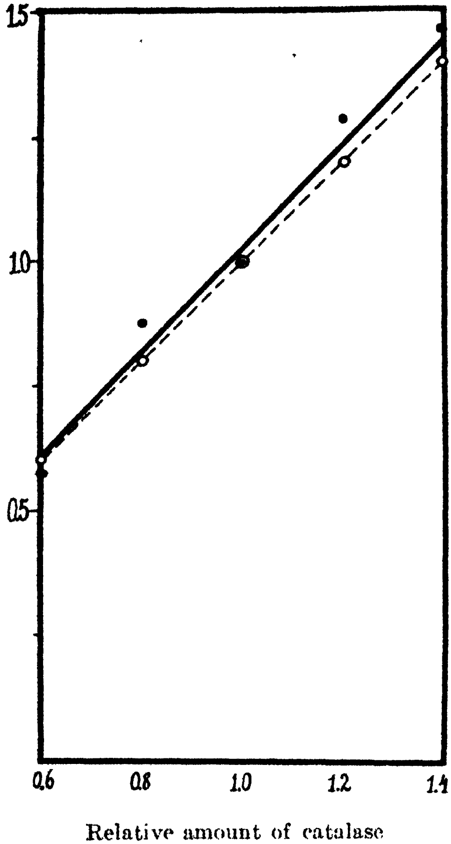

FIG. 3. •—• Relative amount of oxygen set free. ○—○ Relative concentration of catalase.

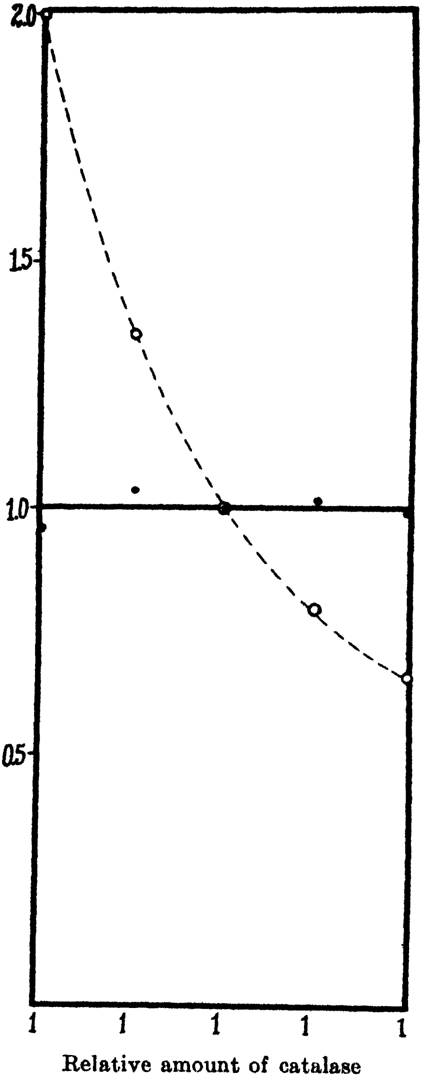

Fig. 4. •—• Relative amount of oxygen set free. ○—○ Relative concentration of catalase.

increase in the oxygen set free. When, however, a constant quantity of peroxide is employed it is found that the degree of its decomposition depends upon the absolute quantity of catalase and that the quantity of oxygen liberated is directly proportional to the catalase. The direct relationship between the varying amounts of catalase and the oxygen given off from a definite

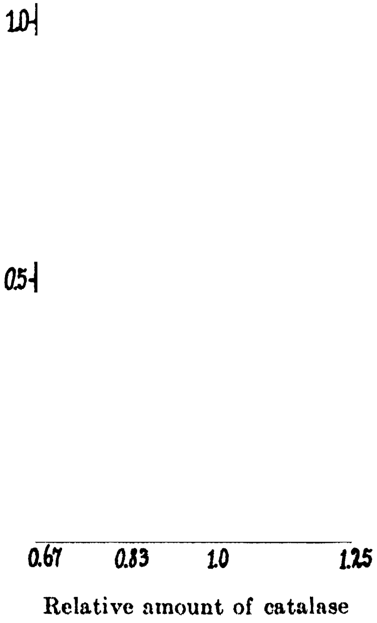

Fig. 5. •—• Relative amount of oxygen set free. ○—○ Relative concentration of catalase.

quantity of hydrogen peroxide is demonstrated by a number of experiments which are graphically recorded in Figs. 3, 4, and 5. These experiments represent a variety of conditions, the quantity and concentration of the catalase varying either separately or simultaneously, while the concentration of the peroxide also is either changed or kept constant. The curves are plotted with the relative quantities of catalase as abscissæ and the corresponding relative quantities of oxygen set free at the time the reaction has been completed as ordinates. The relative concentration of the catalase is also indicated in each graph.

It is clear from these experiments that there is a direct proportionality between the amount of catalase and the amount of oxygen it produces from a given quantity of hydrogen peroxide. This proportionality holds only for the absolute quantity of catalase,[2] changes in concentration alone having no direct effect on the oxygen set free. This furnishes, therefore, additional evidence for the view that the catalase reacts with a definite quantity of hydrogen peroxide.

VIII.

The amount of hydrogen peroxide decomposed (or the amount of oxygen liberated) is a linear function of the quantity of catalase provided a constant amount of hydrogen peroxide is used in the experiments (its concentration need not be constant). With the same quantity of catalase, however, changing the quantity of hydrogen peroxide (or even changing its concentration beyond a certain point) will limit the catalase reaction to a greater or less extent. This, of course, is true only in the case when the hydrogen peroxide is already present in such excess that it is less than 65 per cent decomposed. Within the limits of complete decomposition (100 per cent) and two-thirds decomposition (65 to 70 per cent) the amount of oxygen set free from the hydrogen peroxide increases but not in direct proportion to the increase of its quantity. This can be seen from the data recorded in the first part of Table III as well as from the graph in Fig. 6. On the other hand, when the decomposition of the hydrogen peroxide used falls below 65 per cent the amount of oxygen produced by the same quantity of catalase diminishes with every increase in the hydrogen peroxide. The data in the second part of Table III demonstrate this point. Plotting the results with the relative quantity of hydrogen peroxide as the abscissæ and the relative amount of oxygen formed by the catalase as well as the per cent of decomposition of the peroxide as ordinates, we find that the curve of the oxygen evolution has a smaller slope for every new increase in the amount of hydrogen peroxide employed.

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[2] This rule holds true only when the quantity of catalase is neither too small nor too large for the amount of hydrogen peroxide employed. In these events the disturbing factors already discussed dominate the reaction and there is no longer a direct proportionality between the catalase and the quantity of oxygen liberated from the hydrogen peroxide.

The greatest diminution in the evolution of oxygen from hydrogen peroxide by a certain quantity of catalase occurs, therefore, with the relatively smaller excess of peroxide. This fact is significant because if the diminution were due to an oxidation of the catalase a proportionately greater destruction of it could be expected with the greater amount of hydrogen peroxide.

TABLE III

Hydrogen peroxide		Oxygen evolved from H_2O_2	
Oxygen available.	Relative amount.	Relative amount	Absolute amount
cc			cc.
90	1.00	1.00	85
113	1.26	1.14	97
135	1.50	1.22	104.5
158	1.76	1.37	116
181	2.00	1.43	122.7
112	1.00	1.00	92.0
168	1.50	0.90	82.1
224	2.00	0.83	76.6
448	4.00	0.66	60.8
896	8.00	0.37	34.1
1,344	12.00	0.26	23.7

IX.

The rate of the catalase reaction can be determined by comparing the lengths of time required to liberate a definite quantity of oxygen under different conditions (see Osterhout). The results of a number of experiments were plotted and the time necessary to produce 25, 50, 75, or 100 cc. of oxygen was measured from the graphs. It is understood, of course, that the reaction velocity is the reciprocal of the time. In Table IV the results are given of a number of tests performed under varied experimental conditions. This study extended to the effect upon the reaction rate of constant and varying quantities of catalase (with or with-

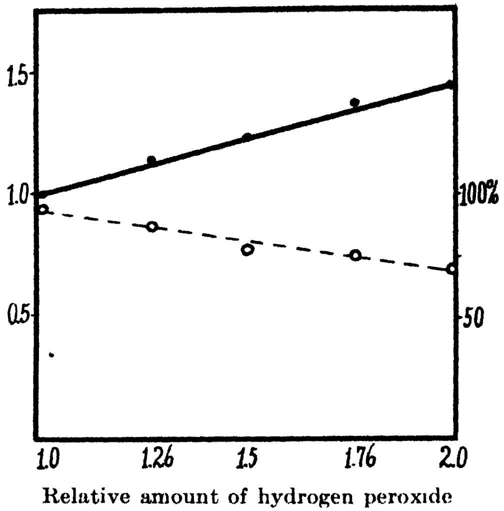


FIG. 6. •—• Relative amount of oxygen set free. ○—○ Per cent of hydrogen peroxide decomposed.

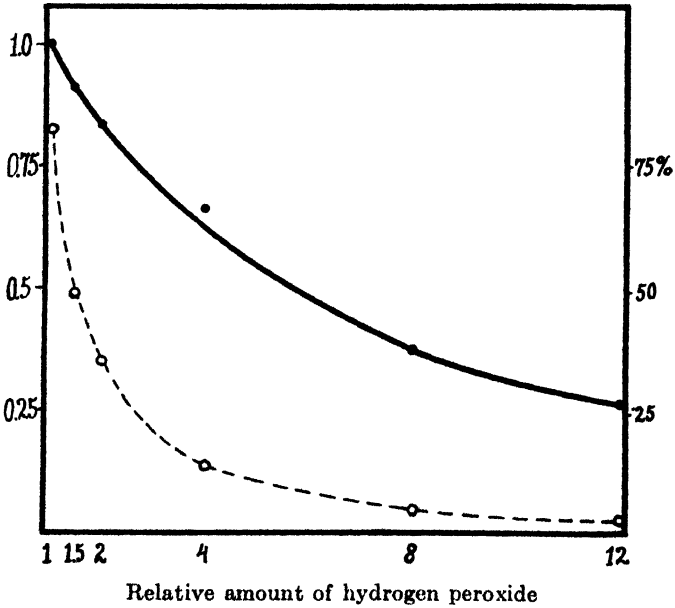


FIG. 7. •—• Relative amount of oxygen set free. ○—○ Per cent of hydrogen peroxide decomposed.

out change of concentration), and of constant and variable quantities of hydrogen peroxide (with or without changing its concentration).

TABLE IV.

Catalase.		Hydrogen peroxide		Time required to evolve oxygen.			
Quantity	Relative concentration.	Concentration.	Oxygen available	25 cc.	50 cc.	75 cc.	100 cc.
cc.		*mol*	*cc.*	*min.*	*min.*	*min.*	*min.*
5	1.0	0.16	90	1.20	3.60	8.5	—
5	1.0	0.20	113	1.55	4.00	8.0	—
5	1.0	0.24	136	1.70	4.25	8.6	18.1
5	1.0	0.28	158	1.85	4.75	8.9	15.8
5	1.0	0.32	181	1.80	4.50	8.8	15.8
5	1.0	0.36	203	1.85	4.60	8.7	15.4
5	1.0	0.40	226	2.35	6.00	11.7	21.7
9	0.67	0.12	100	3.2	9.3	—	
9	0.80	0.14	100	2.9	?	27.0	
9	1.00	0.18	100	2.9	8.5	28.0	
9	1.33	0.24	100	2.9	8.9	27.8	
9	2.00	0.36	100	3.2	9.5	—	
6.75	1.0	0.13	112	1.60	4.10	8.6	
5.63	1.0	0.16	112	1.90	5.20	11.9	
4.50	1.0	0.20	112	2.75	8.20	19.2	
3.75	1.0	0.25	112	3.50	11.40	—	
3.00	1.0	0.30	112	5.50	25.00	—	
3	0.6	0.20	108	2.5	7.2	21.2	—
4	0.8	0.26	145	2.0	5.0	10.7	22.4
5	1.0	0.32	180	1.5	4.0	8.5	15.0
6	1.2	0.39	217	1.3	3.2	5.8	9.5
7	1.4	0.42	254	1.2	2.9	5.5	9.0
3	0.6	0.32	181	4.25	10.75	—	—
4	0.8	0.32	181	1.90	4.85	10.1	21.6
5	1.0	0.32	181	1.50	4.10	8.6	15.2
6	1.2	0.32	181	0.75	2.30	4.5	7.6
7	1.4	0.32	181	0.45	1.60	—	5.5

Increasing the quantity of hydrogen peroxide (also its concentration), while the catalase remains the same, causes a certain amount of retardation of the reaction particularly during the early stages. Thus, in the first experiment the hydrogen peroxide quantity increased from an equivalent of 90 cc. to that of 203 cc. of available oxygen, while the hydrogen peroxide was thus more than doubled, the reaction velocity diminished somewhat, and only 1 minute more was required to liberate 50 cc. of oxygen (4.6 instead of 3.6 minutes). At a later stage in the reaction this difference disappears, and the time required to set free 75 cc. of oxygen is practically the same (about 8.5 minutes). However, when the concentration of the hydrogen peroxide reaches 0.4 gram-molecular the slowing up of the reaction becomes very pronounced and persists through the entire reaction.

When the experiment is performed with varying relative concentrations of catalase (maintaining a constant quantity) while keeping the same quantity of hydrogen peroxide (second experiment), we find that the rate of the reaction remains practically unaltered. To decide whether the constancy of the reaction velocity is due to the fact that the quantity of the hydrogen peroxide or that of the catalase is the same, an experiment was performed in which the relative concentration of the catalase was maintained unchanged through the series (third experiment) while the absolute quantities varied from 3.0 to 6.75 cc. of the extract. The quantity of hydrogen peroxide in the meantime was kept constant. Under these circumstances the rate of the reaction (reciprocal of the time) was found to vary directly with the catalase quantity. The concentration of the catalase is evidently of no particular consequence so far as the reaction velocity is concerned. Two other experiments were made with varying quantities of catalase while both the concentration and quantity of the hydrogen peroxide either varied or were kept constant. Plotting the velocities against the quantities (also the concentrations) of the catalase (Figs. 8 and 9), these results corroborate further the findings of the previous experiments demonstrating definitely that the reaction rate depends directly on the quantity of catalase used, while the effect of the hydrogen peroxide is to limit the rate. This conclusion seems, therefore, a corollary to that derived from the study of the rôle played respectively by the catalase and the hydrogen peroxide in the reaction.

X.

The catalase reaction is generally regarded as belonging to the monomolecular order, only one substance—hydrogen peroxide—undergoing decomposition. Though it is true that the reaction may follow the monomolecular curve, the widely accepted idea that from a dynamic standpoint the catalase reaction is of this particular type requires drastic revision. The evidence presented in this paper goes to show that not only does the hydrogen peroxide disappear but that the catalase as well is used up in the course of the reaction, and that a definite quantitative relation exists between the interacting catalase and hydrogen peroxide. Furthermore, when the time relations of the evolution of oxygen,

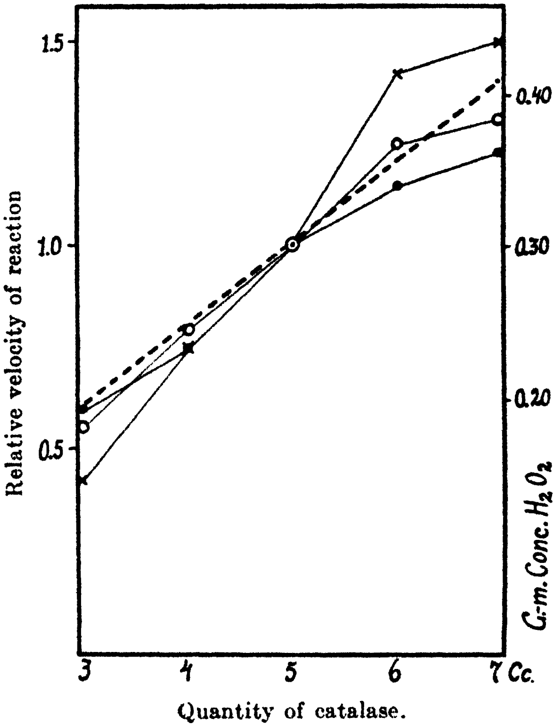
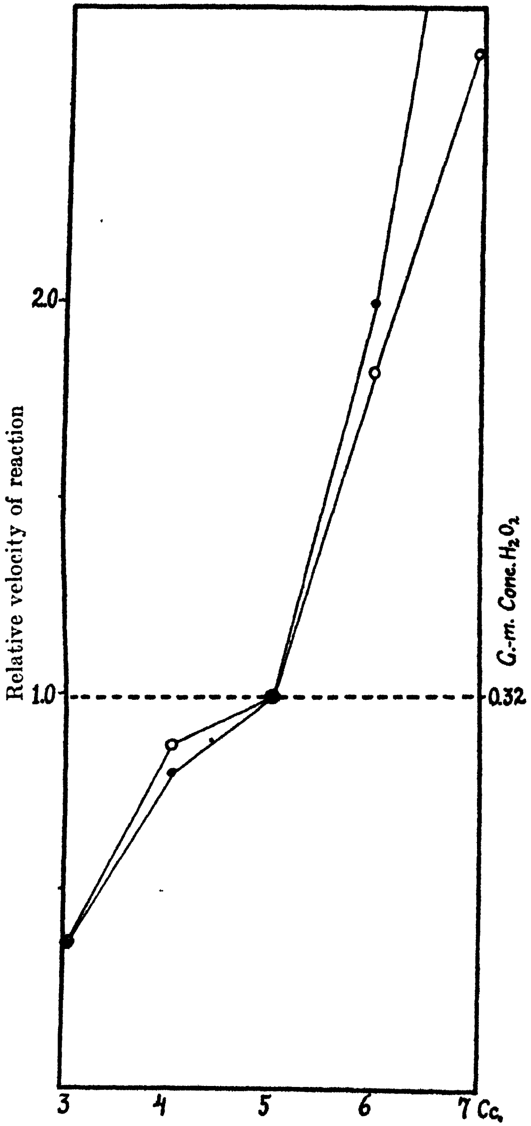


FIG. 8. Effect of changes in catalase on reaction velocity. •—• 25 cc. of oxygen set free. ○—○ 50 cc. of oxygen set free. X—X 75 cc. of oxygen set free.



Quantity of catalase

Fig. 9. Effect of changes in catalase on reaction velocity. The quantity and concentration of hydrogen peroxide is unchanged. •—• 25 cc. of oxygen set free. ○—○ 50 cc. of oxygen set free.

resulting from the reaction of catalase with hydrogen peroxide, are studied under different experimental conditions, and the reaction is followed not for a limited space of time but until the reaction stops, it is soon discovered that it does not invariably follow the monomolecular isotherm. As will be shown presently, the reaction under certain conditions is bimolecular and even one and a half molecular, while under other conditions it does not conform to either of these types. In fact, the course of the reaction seems to depend solely on the quantitative relation between the available catalase and the hydrogen peroxide. Where the former is in excess, the hydrogen peroxide will be completely decomposed; on the contrary, when the hydrogen peroxide is in excess, a greater or less proportion of it will undergo decomposition. Barring from the present discussion either extreme, *i.e.* cases where there is too great an excess of catalase or of hydrogen peroxide, for which I was unable to find a simple mathematical expression of the reaction, we will confine our attention only to such instances where the decomposition of the hydrogen peroxide ranges from about 65 to 100 per cent of the quantity used. Within this limited range the reaction does not run true to any one particular dynamic formula. When 95 to 100 per cent of the oxygen available in the hydrogen peroxide is liberated, the reaction is unquestionably monomolecular, and the reaction coefficients can be determined from the well known formula, $K = \frac{1}{t} \cdot \log \frac{a}{a-x} \cdot$ When the catalase is present in large excess, and this can usually be detected immediately by the extreme vigor and velocity of the reaction, this no longer applies, while there also occur other changes which will be discussed separately in a forthcoming paper. The fact, however, has been definitely established that in the presence of a small excess of catalase the reaction runs true to the monomolecular course. Herein we find the reason why the older observers were led to believe that the reaction is of this particular order. Guided by the erroneous notion that catalase may be destroyed through oxidation by hydrogen peroxide, they experimented with very dilute peroxide solutions which were completely decomposed by the catalase. In other words, the experiments were confined chiefly to a particular range of catalase-peroxide relationship, and this fact was

TABLE V.

Catalase 5 cc. Total volume 50 cc.

Hydrogen peroxide.		Time.	Oxygen evolved.	Reaction coefficient.*	H_2O_2 decomposed.
Oxygen available.	Concentration.				
cc.	*mol*	*min.*	*cc.*	$K_1 \times 10^4$	*per cent*
90	0.16	5	64.4	1092	95
		10	81.6	1046	
		12	85.0	1030	
				1056	
				$K_2 \times 10^1$	
113	0.20	5	63.1	95	86
		10	85.6	97	
		15	95.1	94	
		18	97.0	87	
				93	
				$K_3 \times 10^5$	
135	0.24	5	58.5	107	76
		10	82.5	113	
		15	96.5	121	
		20	103.3	116	
		24	104.5	112	
158	0.28	5	56.7	67	73
		10	81.6	66	
		15	99.5	71	
		20	110.4	72	
		25	115.2	67	
		27	115.9	69	
181	0.32	5	56.5	50	68
		10	81.6	46	
		15	99.5	46	
		20	110.6	44	
		25	118.0	42	
		30	122.0	39	
		34	122.7	44.7	

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* The designations K_1, K_2, and K_3 are employed as abbreviations for the coefficients of unimolecular, one and a half molecular, or bimolecular reactions, respectively.

TABLE V—*Concluded.*

Hydrogen peroxide		Time	Oxygen evolved	Reaction coefficient.	H_2O_2 decomposed.
Oxygen available.	Concentration.				
cc.	*mol*	*min*	*cc*		*per cent*
203	0.36	5	56.0		58
		10	82.9		
		15	100.5		
		20	111.0		
		25	117.1		
		30	119.0		
226	0.40	5	47.7		49
		10	69.4		
		15	86.6		
		20	97 8		
		25	105.2		
		30	109.1		
		33	109.7		

responsible for the unqualified assumption that the catalase reaction is of this particular type.

When, however, the catalase-hydrogen peroxide ratio is so adjusted that less than 95 per cent of the available oxygen is set free, the reaction is no longer monomolecular. Either by diminishing the quantity of catalase or by increasing the quantity of hydrogen peroxide any degree of decomposition may be secured, as was already expounded in the foregoing. When the decomposition is somewhere between 85 and 95 per cent of the hydrogen peroxide (the limits are not sharply defined) the course of the reaction follows the curve of a one and a half molecular, and the reaction coefficient can be determined from the formula, $K = \frac{1}{t}\cdot\frac{\sqrt{a}-\sqrt{a-x}}{\sqrt{a(a-x)}}$. When the decomposition is somewhere between 70 and 80 per cent of the hydrogen peroxide used in the experiment the reaction runs true to the bimolecular course, and $K = \frac{1}{t}\cdot\frac{x}{a(a-x)}$ applies within these limits. Since it has already been shown that when the decomposition of the hydrogen peroxide falls below 65 per cent the actual amount of oxygen set free by the catalase as well as the reaction velocity are considerably diminished, it is obvious that we are dealing not simply with a bimolecular reaction but also one that is apparently reversible.[3]

Before discussing this matter further a few experiments will be recorded in which either a constant quantity of catalase was used with varying amounts of hydrogen peroxide or a constant quantity of hydrogen peroxide while the catalase was varied.

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[3] From theoretical considerations Yamazaki also comes to the conclusion that depending upon the relative amounts of hydrogen peroxide and catalase the type of the reaction may shift from the bimolecular to the monomolecular. In his calculations he nevertheless employs the equation for a monomolecular reaction. This he does even where, as will be shown, the equation does not apply. Recalculating his data given in Table 76 (IV and V) where with 10 and 5 cc. of the catalase preparation, respectively, he obtained 90 and 75 per cent of decomposition of the hydrogen peroxide, it can be shown that the reaction follows the equation of either a one and a half or of a bimolecular reaction as was also found to be the case with my preparation of liver catalase. I may add that since the paper had been sent to press I had occasion to experiment with catalase preparations from different sources and was able to substantiate the results in every instance. In the tabulation below the values of the constant (*K*) as found by Yamazaki with the aid of the monomolecular formula and those which I calculated in accordance with the bi-, or one and a half molecular equation are set down side by side. The point is brought out so clearly that no further comment is required.

	Time.	*A*	*X*	*K* (monomolecular).	*K* (bimolecular).
	min.				
5 cc. catalase, 75 per cent decomposition.	0	12.52			
	5.08	12.52	2.20	0.0159	0.00335
	9.58	12.52	3.58	0.0145	0.00334
	15.16	12.52	4.80	0.0114	0.00328
	20.83	12.52	5.87	0.0114	0.00339
	26.08	12.52	6.54	0.0088	0.00335
					***K* (One and a half molecular).**
10 cc. catalase, 90 per cent decomposition.	0	13.33			
	4.03	13.33	3.24	0.0357	0.0102
	13.11	13.33	8.72	0.0276	0.0113
	18.46	13.33	9.18	0.0245	0.0119
	23.86	13.33	10.11	0.0204	0.0118

The results under both kinds of conditions are the same. The experiments were all made under a uniform temperature (20–21°C.) and similar hydrogen ion concentration of the medium (pH = 6.7 to 6.9).

TABLE VI.

Catalase 4.5 cc.

Hydrogen peroxide.		Time	Oxygen evolved	Reaction coefficient.	H_2O_2 decomposed.
Oxygen available.	Concentration				
cc.	*mol*	*min.*	*cc.*	$K_1 \times 10^4$	*per cent*
87	0.2	5	36.5	472	100
		10	54.3	425	
		15	66.0	412	
		20	74.2	416	
		30	82.3	423	
		40	86.2	509	
		45	87.1		
				443	
				$K_3 \times 10^5$	
112	0.2	5	36.7	88	82
		10	53.5	82	
		15	67.0	89	
		20	75.8	93	
		30	85.8	97	
		40	91.2	98	
		45	92.0		
				91	
168	0.2	5	36.4		49
		10	53.4		
		20	74.5		
		30	80.7		
		35	82.1		
224	0.2	10	49.6		34
		20	67.6		
		30	74.9		
		35	76.6		

A review of these data shows that the results may be calculated in accordance with different dynamic formulas depending upon the degree of decomposition of the hydrogen peroxide effected in the reaction. The results of the four series of experiments are presented diagrammatically in Fig. 10.

TABLE VII.

Hydrogen peroxide equivalent to 112 cc. O_2.

Catalase.	Time.	Oxygen evolved	Reaction coefficient	H_2O_2 decomposed.
cc.	*min.*	*cc.*	$K_1 \times 10^4$	*per cent*
6.75	5	56.6	620	100
	10	80.1	545	
	15	94.1	531	
	20	102.1	559	
	25	106.3	517	
	30	108.8	515	
	40	111.8		
			548	
			$K_2 \times 10^4$	
5.63	5	49.0	63	93
	10	69.3	59	
	15	82.1	59	
	20	91.5	63	
	25	97.5	67	
	30	101.0	69	
	35	103.7		
			61.7	
			$K_3 \times 10^5$	
4.5	5	37.4	90	79
	10	55.1	88	
	15	67.4	90	
	20	76.1	95	
	25	80.7	92	
	30	83.7	88	
	35	85.8	84	
	45	88.0		
			90	
3.75	10	47.1		63
	20	63.5		
	30	69.2		
	40	70.0		
3.0	10	33.8		51
	20	46.4		
	30	53.2		
	40	56.4		
	45	57.2		

We can recognize (Fig. 10) three distinct zones in the diagram corresponding to 68 to 82 per cent decomposition, 88 to 93, and 95 to 100 per cent. A different formula applies for each zone, and for this zone alone. We may consider, therefore, that the catalase reaction is bimolecular changing to monomo-

TABLE VIII

Hydrogen peroxide equivalent to 181 cc. of O_2.

Catalase.	Time.	Oxygen evolved	Reaction coefficient	H_2O_2 decomposed
cc.	*min.*	*cc*	$K_1 \times 10^4$	*per cent*
7	5	88.7	573	100
	10	131.0	560	
	15	155.2	567	
	20	169.1	596	
	25	176.7	662	
	30	180.7	592	
			$K_2 \times 10^4$	
6	5	78.2	49	88
	10	112.2	46	
	15	135.0	49	
	20	147.0	49	
	25	153.4	47	
	30	157.0	44	
	34	158.3	47.3	
			$K_3 \times 10^5$	
5	5	56.5	50	68
	10	81.6	46	
	15	99.5	46	
	20	110.6	44	
	25	118.0	42	
	30	122.0	39	
	33	122.7	44.7	
4	10	74.8		59
	20	98.0		
	30	105.5		
	33	106.3		
3	10	47.6		39
	20	66.6		
	30	71.2		

lecular, the middle zone merely representing the transition from the one to the other. Mellor recites a number of instances where the reaction shows a similar change. He says[4] that "the gradual approach of the velocity curves for the bimolecular reaction to the curves for a unimolecular reaction as the amount of one of the reacting components of the bimolecular reaction is increased, shows very clearly how the course of a bimolecular reaction might appear unimolecular when one of the reacting components is in excess." Among reactions of this kind Mellor mentions the reduction of potassium permanganate with an excess of oxalic acid; the action of an excess of hydrogen peroxide on hydriodic acid; etc. The catalase reaction seems to belong to the same category except that in this instance it is not the excess of hydrogen peroxide but of the catalase which shifts the reaction from the order of a bimolecular to that of a unimolecular.

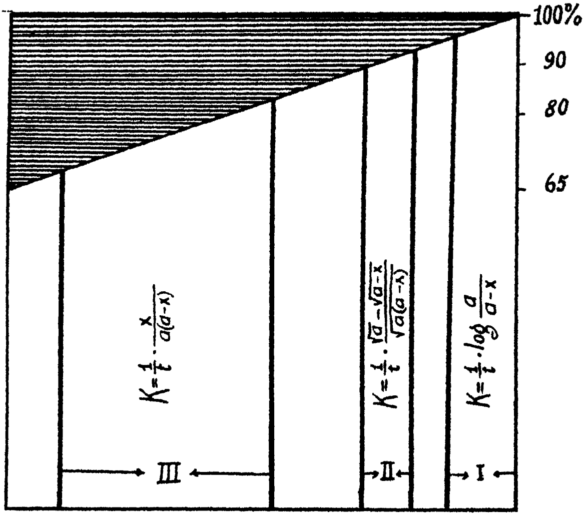
100%
90
80
65
$K=\frac{1}{t}\cdot\frac{x}{a(a-x)}$
$K=\frac{1}{t}\cdot\frac{\sqrt{a}-\sqrt{a-x}}{\sqrt{a(a-x)}}$
$K=\frac{1}{t}\cdot\log\frac{a}{a-x}$
III
II
I

FIG. 10. Diagram showing the order of reaction depending on the degree of decomposition of hydrogen peroxide. The shaded area represents the undecomposed fraction of hydrogen peroxide.

Evans in a very thorough study of the catalase reaction (which he regards simply as a catalytic decomposition of hydrogen peroxide) observed that the reaction coefficients, calculated according to the formula $\frac{dt}{dx} = K\ (a-x)$, showed a peculiar behavior. In some instances the values of K would form an ascending, sometimes a descending series, and only occasionally did he obtain fairly constant values. He distinguished, therefore, three periods: the rectilinear, the infralogarithmic, and the logarithmic, a terminology which does not seem happily chosen. The latter is the one when the reaction really follows the curve of a monomolecular reaction. Examining his data, I find that this condition was rarely met, and only when the substrate (H_2O_2) was used in very dilute solution while the amount of enzyme was "not very small." In other words, when there was enough catalase to completely decompose the hydrogen peroxide. The three phases which Evans noted are all related to the catalase-peroxide ratio. I found that the same three phases occur no matter whether the reaction follows the monomolecular, bimolecular, or an intermediate course. Whenever the catalase is present in too great an excess the values of K (monomolecular) will continually increase. On the other hand, when there is less catalase than is necessary to decompose the entire amount of peroxide the values of K gradually diminish. This is likewise true when the values of K are calculated for a one and a half or for a bimolecular reaction. The change of phase is brought about very readily by the smallest alteration in quantity of either the catalase or the hydrogen peroxide. With a little experience it is possible to adjust the quantities so as to make the reaction proceed according to any of the three formulas employed in this paper for calculating the reaction coefficient. It is remarkable how little it is necessary to change the quantities to produce appreciable differences in the values of K. Sometimes a change by a few drops of the catalase preparation was quite sufficient to bring this result about. When one bears in mind that with a decomposition of the hydrogen peroxide of about 75 to 80 per cent the reaction follows almost ideally the bimolecular curve, it is a simple matter indeed to adjust conditions so that the results of an entire experimental series can be made really comparable. In the study of the effect of the concentration of hydrogen ions upon the reaction it was already pointed out that the respective quantities of both the catalase and the hydrogen peroxide were so chosen that the reaction was typically bimolecular. It is by virtue of this adjustment that it was possible to demonstrate in that series that increasing the hydrogen ion concentration not only limits the decomposition of hydrogen peroxide and slows up the reaction, but that the type of the reaction also changed and it no longer followed the course of a bimolecular curve when the pH fell below 6.0.

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[4] Mellor, p. 42.

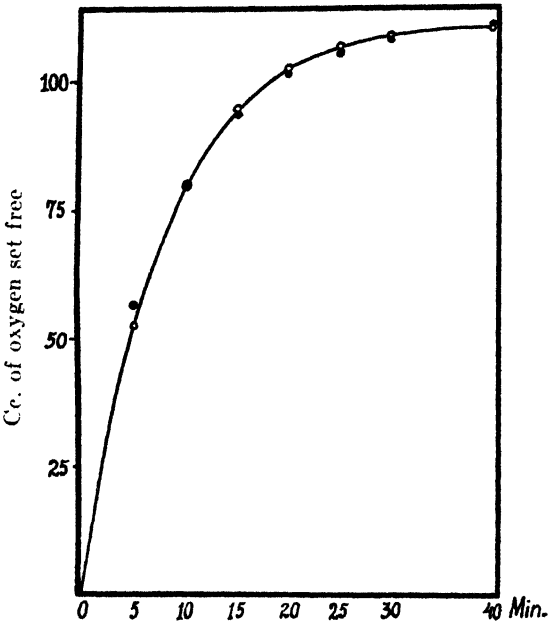

FIG 11. Table VII, Experiment 1. •—• Experimental results. ○—○ Calculated results.

In Figs. 11, 12, and 13, the experimental curves are plotted together with the theoretical curve calculated with the aid of the average value of K for each respective experiment. The similarity of the two curves requires no further comment.

Before closing the paper it may be well to point out the bearing of the results upon the question of the technique of catalase determinations. The use of very large quantities of hydrogen peroxide (equivalent to 500 or 600 cc. oxygen), as is practised commonly in researches on catalase, is objectionable because the depressing effect is great unless very large amounts of the catalase preparation are employed. There is no obvious advantage in working with such tremendous quantities. On the other hand, when an attempt is made to compare the catalase activity of preparations of presumably different strengths, the depressing effect will be much greater in the case of the weaker sample and under such conditions the tendency will be to exaggerate the differences. In fact the entire method of comparing several samples of catalase on the basis of the amount of oxygen which they respectively liberate from hydrogen peroxide is of questionable accuracy. The comparison should instead be made between respective quantities of catalase preparation required to set free the same amount of oxygen from a given quantity of hydrogen peroxide. It is further advisable to adjust the reaction to follow some definite course (a 75 per cent decomposition of the hydrogen peroxide is a very good basis). Although the oxygen formation

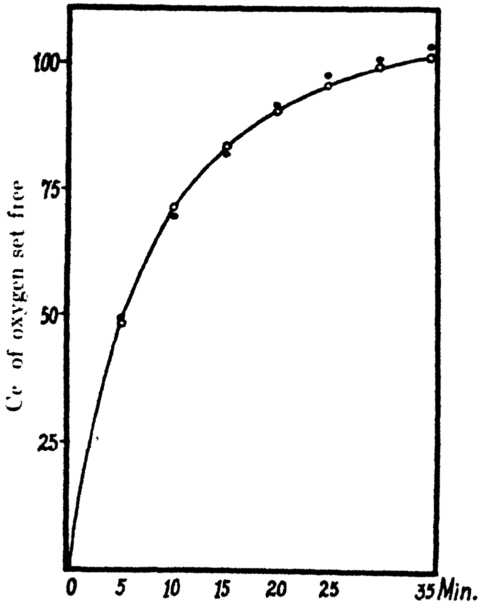

FIG. 12. Table VII, Experiment 2. •—• Experimental results. ○—○ Calculated results.

is a linear function of the quantity of catalase, this rule does not hold true when either the catalase or the hydrogen peroxide are in great excess. When, however, the catalase is varied to produce a certain degree of decomposition with the same quantity of peroxide, the catalase strengths will be inversely proportional to the quantities used for the tests. The method of estimating the catalase strength followed in most investigations on catalase is so crude and untenable from a chemical standpoint that one naturally is reluctant to accept the conclusions drawn from those researches, especially where the conclusions are of far reaching significance.

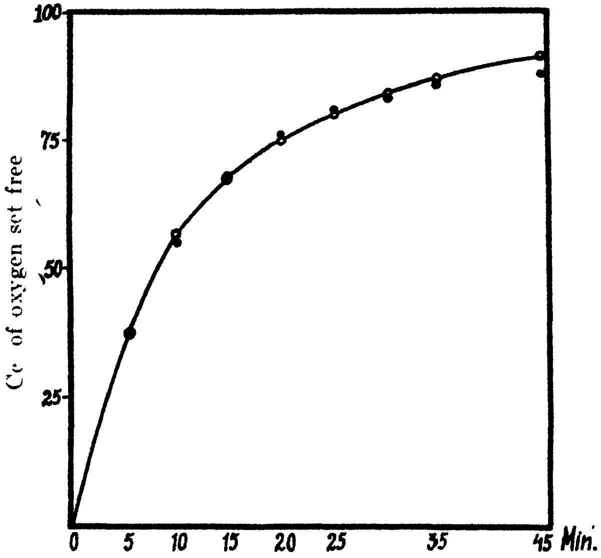

Fig. 13. Table VII, Experiment 3. •—• Experimental results. ○—○ Calculated results.

Another important matter is the fact that in the catalase experiments the reaction is allowed to go on for a set length of time, usually 10 or 15 minutes. The reasons for determining relative reaction speeds from the time periods required to effect a given result, rather than from the results effected in a given time period have been fully discussed by Osterhout, and need not be repeated. It should be noted, however, that when the reaction is permitted to run for 10 minutes, only 50 or perhaps 60 per cent of the reaction is completed. For comparative purposes, it may be argued, this will not matter, and this might be true if the reaction were always brought to the same end-point. This is not likely to be the case. If, on the other hand, the reaction is so vigorous and rapid as to be completed within the 10 or 15 minutes it is practically certain that there is a great excess of catalase. In this event, of course, the oxygen evolution will again fail to give a correct measure of the relative catalase strengths inasmuch as there is no means of determining whether or not the relative excess is the same in each instance. Little credence can therefore be given to results of catalase experiments unless very large differences are demonstrated.

BIBLIOGRAPHY.

Euler, H., *Beitr. chem. Physiol. u. Path.*, 1906, vii, 1.
Evans, C. A. L., *Biochem. J.*, 1907, ii, 133.
Hampton, H. C., and Baas-Becking, L G. M., *J. Gen. Physiol.*, 1920, ii, 635.
Iscovesco, H., *Compt. rend. Soc. biol.*, 1905, lvii, 1055.
Mellor, J. W., Chemical statics and dynamics, London, 1904.
Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1913, liii, 320.
Osterhout, W. J. V., *Science*, 1918, xlviii, 172.
Senter, G., *Z. physik. Chem.*, 1903, xliv, 257.
Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
Yamazaki, E., *Tohoku Imperial Univ., Scient. Rep.*, 1920, iii, No. 13.

THE RELATION OF THE MIGRATION OF IONS BETWEEN CELLS AND PLASMA TO THE TRANSPORT OF CARBON DIOXIDE.*

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(Received for publication, May 23, 1921.)

Due to the interest attached to the relationship of the chloride-bicarbonate equilibrium (Henderson, 1921) to the transport of carbon dioxide by the blood, we are publishing some of our data on this subject. Van Slyke and Cullen (1917) showed that the plasma of whole blood which had been shaken with varying proportions of carbon dioxide had lost an amount of chloride which was sufficient to account for about two-thirds of its increase in bicarbonate. More recently, Fridericia (1920) has published data which show that the loss of chloride more nearly accounts for the gain in bicarbonate. Henderson and coworkers (1920) found that only 60 per cent of the increase of bicarbonate was due to the migration of chlorine.

A study of this subject and permeability of cells in general were uppermost in mind when one of us started the adaptation of methods of inorganic analysis 3 years ago.[1] At that time the existing methods of determining sodium and potassium were laborious and required excessive amounts of material. We are now able to make a study of the permeability of cells to the more important anions and cations on small samples of blood.

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* The data in this paper are taken from a thesis presented by Miss Eaton in partial fulfillment of the requirements for the degree of Master of Science, Washington University, 1921.

[1] A study of methods of analysis of inorganic substances of biochemical importance was begun in the fall of 1918 by R. D. Bell and the senior author of this paper. Procedures for the determination of sodium and phosphorus have been published. Complete details of the systematic inorganic analyses of blood will soon be submitted to the Journal for publication.

Practically all the investigators in this field have admitted the permeability of red cells to hydrochloric acid when the blood is treated with carbon dioxide. According to Hamburger (1916) and coworkers this effect is extended to include sulfates, phosphates, sodium, potassium, and calcium. A number of investigators (Gürber, 1895) have failed to find an exchange of cations between cells and plasma. Lately, Van Slyke (1921) and Henderson and coworkers (1920) have stated that the migration of cations probably plays no part in the transport of carbon dioxide. While we are not in a position to state emphatically that there is *no transfer*, our analytical data show clearly that quantitatively the shift, if it occurs at all, *is very small.*

Methods.

For our investigation of this problem we have devised a scheme of inorganic analysis which permits the determination of sodium, potassium, chlorine, and phosphorus on 10 cc. of serum. An additional quantity of 3 cc. is necessary for bicarbonate determinations.

Briefly the method is: Transfer 10 cc. of serum to a 100 cc. flask containing about 70 cc. of distilled water. Add while shaking 10 cc. of 20 per cent trichloroacetic acid.[2] Make to mark and mix well. After 30 minutes filter through dry paper. Since the filtration is rather slow, it is essential that both the funnel and flask be covered to avoid evaporation. Usually we obtain about 83 cc. of filtrate of which the following amounts are used for the determinations named below.

As may be seen from Table I we have utilized only 50 cc. of our filtrate; the remainder may be used for a repetition of doubtful analyses. We expect to extend this scheme to the determination of calcium and magnesium.

[2] We had already worked out independently the use of trichloroacetic acid for chloride analysis when Smith's (1921) paper appeared. Our results on these filtrates agree very closely with values obtained on picric acid filtrates from blood plasma. Invariably, however, the latter procedure gives results, which are 2 or 3 per cent higher on whole blood. We have not found time to study this intensively and are still a little undecided which method is the more nearly correct.

Treatment of the Blood.—In order to avoid as far as possible the effect of change in strength (as an acid) of the hemoglobin in passing from the oxidized to reduced form, we have generally maintained in our saturators a sufficient amount of oxygen to keep essentially all of the hemoglobin as oxyhemoglobin. When the higher tensions of carbon dioxide were used, oxygen was added from a tank to maintain its concentration between 20 and 21 per cent. Barcroft (1914) has shown that about 13 per cent of oxygen is sufficient to keep over 90 per cent of the hemoglobin in the oxidized form in the presence of 11 per cent of carbon dioxide. We think that 20 per cent oxygen was sufficient to prevent the reduction of more than 10 per cent of the oxyhemoglobin in all of our experiments in which the gas mixture contained not more than 20 per cent carbon dioxide.

TABLE I

Analysis	Volume of filtrate used	Method
	cc.	
Sodium	10	Sodium cesium bismuth nitrite, Doisy-Bell (1921).
Potassium ...	20	Cobalti-nitrite.
Chlorine	10	McLean-Van Slyke solutions (1915).
Phosphorus.....	10	Colorimetric, Bell-Doisy (1920).
Total...........	50	

Beef blood was defibrinated and filtered through several layers of gauze, and 60 cc. samples were measured out into a series of 2½ liter bottles. At least 6 liters of the gas mixtures were passed through each bottle and the openings closed with pinch-cocks. The inlet tube extended nearly to the bottom of the bottle, the outlet being flush with the stopper. As our chief aim was to study the shift of inorganic ions from serum to corpuscles under higher tensions of CO_2, we did not analyze the gas mixtures in the saturators in any except the last experiment. These analyses revealed the fact that 6 liters were insufficient to bring the gas to even the approximate per cent of CO_2 given in our tables. Our 20 per cent CO_2 mixture probably sufficed to make the gas in the saturator not over 15 per cent CO_2. The bottles were rotated to secure a maximum surface for the rapid attainment of equilibrium. After agitation for about 20 minutes the blood was drawn off under oil into centrifuge tubes.

For the determination of corpuscle volume, we used graduated tubes of 6 mm. bore. All samples of the blood were centrifuged simultaneously for 30 minutes at 3,500 R.P.M. immediately after equilibration. Our centrifuge was occasionally tested to show that this speed was actually attained. The volume of corpuscles was read and the tubes were again centrifuged for 15 minutes. If comparison showed that any appreciable decrease had occurred the tubes were centrifuged again. The serum was immediately removed for analysis.

The various analyses were started and the bicarbonate determinations finished on the same day that the blood was taken. One of us (E. P. E.) determined the chlorides; the other (E. A. D.), the bicarbonates.

In general, the bicarbonate determinations were made according to the technique described by Van Slyke and coworkers (1919), using a standard pH 7.2 and phenolsulfonephthalein. It seemed preferable to titrate to a standard pH than to use the various gas mixtures required for this purpose if the Van Slyke-Cullen (1917) method were used. This point is mainly theoretical in nature, but will assume some importance in the larger changes of bicarbonate concentration.

We are giving one of our experiments in its entirety at this point to aid in the discussion of our results. Other similar data are given in the protocols to strengthen our conclusions.

Corpuscle Volume.—By reference to Table II it can be seen that the volume of corpuscles increased with increasing tensions of CO_2. This effect which was emphasized by Hamburger (1916) has not been confirmed by Joffe and Poulton (1920). The reason for their exceedingly variable results is not clear to us. In none of our experiments have we failed to find an increase in corpuscle volume with increasing tensions of CO_2. This is probably due to the fact that the osmotic pressure of the cell contents is increasing more rapidly than that of the serum. The serum does not show a net gain of ions; for each molecule of $NaHCO_3$ gained a molecule of HCl is lost. The corpuscles, however, gain this molecule of HCl.

TABLE II.

Experiment 6.—Defibrinated beef blood equilibrated with various air-carbon dioxide mixtures at 25°C. Centrifuged for 45 minutes.

No hemolysis. Bicarbonate by titration. Figures in column headed, CO_2, signify the amount of carbon dioxide in the 6 liters of gas passed through the saturators.

Treatment of whole blood.	Corpuscles	NaCl		$NaHCO_3$ concentration.	Na per 100 cc	K per 100 cc	P per 100 cc
		Per 100 cc	Concentration				
CO_2 per cent	*per cent*	*mg.*	*mol*	*mol*	*mg*	*mg*	*mg.*
3	42 9	623	0.1068	0 0293	322	20.9	7 9
10	43.1	610	0 1043	0.0317	319	21.3	7.8
20	43.5	602	0.1030	0.0345	326	21 3	7.8
100	45.5	564	0.0965	0.0431	345	22 9	7.8

Correction for change in volume of corpuscles.

10 per cent $\frac{571}{569} \times 0.0293 = 0.0294$ M $NaHCO_3$ expected.

$\times\ 0.1068 = 0.1071$ M NaCl "

20 per cent $\frac{571}{565} \times 0.0293 = 0.0296$ M $NaHCO_3$ "

$\times\ 0.1068 = 0.1079$ M NaCl "

100 per cent $\frac{571}{545} \times 0.0293 = 0.0307$ M $NaHCO_3$ "

$\times\ 0.1068 = 0.1119$ M NaCl "

Effect of increasing CO_2 content of air from:

	3 to 10 per cent		3 to 20 per cent		3 to 100 per cent	
	BCl	*$BHCO_3$*	*BCl*	*$BHCO_3$*	*BCl*	*$BHCO_3$*
	mol	*mol*	*mol*	*mol*	*mol*	*mol*
Before increase.	0.1071	0.0317	0.1079	0.0345	0.1119	0.0431
After "	0.1043	0.0294	0.1030	0.0296	0.0965	0.0307
	−0.0028	+0.0023	−0.0049	+0.0049	−0.0154	+0.0124

This molecule of hydrochloric acid probably reacts with the alkali oxyhemoglobinate in much the same way that the carbonic acid does.

$$
\begin{array}{rcl}
HCl & \rightleftharpoons & Cl^- + H^+ \\
BHbO & \rightleftharpoons & B^+ + HbO^- \\
 & & \quad\ \downarrow\uparrow \\
 & & B^+ + Cl^- + HHbO
\end{array}
$$

Instead of two we now have three units which exert osmotic pressure. This means a real gain and quite naturally water passes from the serum into the cells to equalize the osmotic pressure on both sides of the membrane.

When the hydrogen ion concentration passes the isoelectric point of oxyhemoglobin we have a very similar phenomenon. There is no net gain of ions in the serum but the increase continues in the cell due to the hydrochloric acid gained. This acid probably reacts with a molecule of hemoglobin which is now functioning as a base. Whereas, previously we had only an undissociated hemoglobin molecule, we now have two ions.

$$\mathrm{HbO} + \mathrm{HCl} \rightleftharpoons \mathrm{HbO.HCl} \rightleftharpoons \mathrm{HbO.H^+} + \mathrm{Cl^-}$$

These schematic equations are intended to express only the increase in osmotic pressure of the cells. We do not intend that they shall convey our idea of the linkages between acids or alkalies and proteins. Other factors such as alterations of the degree of association of hemoglobin molecules may influence the changes of osmotic pressure.

It seems evident to us that the cause of swelling of the corpuscles is the increase of osmotic pressure within the cell. We should then expect water to pass in until both the serum and cell contents have the same osmotic pressure. The volume of serum per 100 cc. of blood is now less than formerly. All its constituents, provided no migration has occurred, should be present in greater concentration. According to this view, it is essential that corrections be introduced in our calculations for the change in volume of the corpuscles.

Reference to Table II shows that at 20 per cent CO_2, the corpuscle volume has increased 0.6 cc. per 100 cc. of whole blood. This amounts to slightly more than 1 per cent increase in the concentration of the serum salts. The serum volume at 3 per cent CO_2 is 57.1 cc.; at 20 per cent CO_2 it is 56.5 cc. Consequently the concentration of sodium, potassium, bicarbonate, chlorine, and phosphorus at 3 per cent CO_2 must be multiplied by $\frac{571}{565}$ to give the expected concentration at 20 per cent.

$\frac{571}{565} \times 0.0293 = 0.0296$ M $NaHCO_3$ expected value.

$\frac{571}{565} \times 0.1068 = 0.1079$ M NaCl " "

Gain in base by loss of HCl, $0.1079 - 0.1030 = 0.0049$ M
" " " as $NaHCO_3$, $0.0345 - 0.0296 = 0.0049$ M

We have purposely taken an experiment where the agreement is better than usual. If we have failed to correct for the volume change of corpuscles the loss in chloride would account for only 73 per cent of the gain in bicarbonate.

Gain in base by loss of HCl, $0.1068 - 0.1030 = 0.0038$ M
" " " as $NaHCO_3$, $0.0345 - 0.0293 = 0.0052$ M

The foregoing calculations demonstrate the error caused by failure to correct for the loss of water from the serum. The discrepancy, of course, will be much larger in experiments where the corpuscle volume change is larger; *i.e.*, at 100 per cent CO_2.

It is evident that mathematically the accurate determination of corpuscle volume is of great importance if one wishes to study the true relationship between the direct and indirect methods of determination of increased base in plasma. For instance, if the volume of corpuscles found had been 44.1 per cent instead of the 43.5 per cent then our calculations would have been:

$\frac{571}{559} \times 0.0293 = 0.0299$ M $NaHCO_3$ expected value.

$\frac{571}{559} \times 0.1068 = 0.1091$ M NaCl " "

Gain in base by loss of HCl, $0.1091 - 0.1030 = 0.0061$ M
" " " as $NaHCO_3$, $0.0345 - 0.0299 = 0.0046$ M

From a very close agreement between the values, an error of about 1 per cent in determination of plasma volume produces results which are rather widely divergent.

The foregoing series of calculations illustrates why the previously published figures have shown that the shift of hydrochloric acid freed an amount of base equivalent to only about two-thirds or three-fourths of the increase of bicarbonate. When we started our work we fully expected to find the remainder accounted for by a passage outward of sodium from the corpuscles.

However, we now feel that such an explanation is entirely superfluous.

These results make it appear more than probable that in as far as the plasma functions as a carrier of carbon dioxide (Joffe and Poulton, 1920; Smith, Means, and Woodwell, 1921; and Fridericia, 1920) the transport is based entirely (providing the $[H^+]$ of the plasma remains constant) upon the passage of hydrochloric acid back and forth across the cell membrane. That this mechanism is possible is closely related to the preeminent buffer value of hemoglobin due to its change of dissociation constant in passing from oxyhemoglobin to reduced hemoglobin.

Permeability of Blood Corpuscles.

We have found it quite easy to repeat the demonstration of the permeability of cells to the chloride ion. Naturally one would expect this effect to be extended to include other anions. However, when one considers the concentration of the other inorganic anions in serum ($P = 0.002$ M; $S = 0.002$ M) it is evident how little importance they would probably have in the transport of carbon dioxide.

Though we have not attempted to decide upon the permeability to the sulfate ion, we have made a few colorimetric determinations of inorganic phosphate. Although nearly all of our results point to a migration of phosphate they are not decisive in that most of the differences are within the possible experimental error. Our one gravimetric determination indicates that no shift of inorganic phosphate occurs. We are very loth to draw any conclusion from this but will extend our work in this direction.

With respect to a shift of cations, Hamburger (1916) concluded from the results of a few experiments that carbon dioxide causes a passage of potassium into the cells and of sodium outward into the plasma. Previous workers (Gürber, 1895) had failed to detect any shift of cations but Hamburger attributed this to a lack of suitable methods of analysis.

We fully expected to detect a transfer of both sodium and potassium. Quantitatively, Hamburger found that shaking horse blood with 20 volumes per cent of CO_2 (20 cc. of pure CO_2 to 80 cc. of blood) caused about 17 per cent of the potassium to pass into the corpuscles and a 6 per cent increase of the sodium of the serum. While our experiments were not conducted exactly as Hamburger's were, we have a few data on blood treated in a comparable manner. We have shaken beef blood with 40 or 50 volumes of gas, containing proportions of CO_2 varying from 5 to 100 per cent. In practically no case has any shift of either potassium or sodium been found.

We consider that our determinations of sodium have more significance than those of potassium. In case our duplicate results varied by more than 2 per cent other analyses were made. In general the values reported are the mean of two results which varied by less than 2 per cent. Such being the case a variation of 2 per cent of the value found from that expected may be considered a real shift. In some of our later experiments a deviation of 1 per cent may be taken as an exchange of sodium. By reference to the protocols it can be seen that only very infrequently does a shift of sodium seem to occur. In view of its rare occurrence and the difficulty of obtaining filter paper, etc., free from sodium we are inclined to ascribe this to experimental error.

Our determinations of potassium are less reliable than those of sodium. However, it is possible that any variation greater than from 2 to 3 per cent from the expected value is a real transfer of potassium. This rarely occurs.

We feel that our results lead to the conclusion that blood cells are permeable to the chloride ion but impermeable to both sodium and potassium. Collip (1921) has recently published data which also point in this direction.

DISCUSSION.

According to Van Slyke's (1921) recent review, hemoglobin seems to be by far the most important factor in the transport of carbon dioxide from the tissues to the lungs. As it is non-diffusible an auxiliary reaction is utilized to assist the plasma to take up or give off carbon dioxide. This mechanism is a shifting back and forth across the cell membrane of hydrochloric acid. As we picture the process it is dependent upon a very slightly varying hydrogen ion concentration of the plasma.

In the capillaries where the tension of carbon dioxide is high the hydrogen ion concentration of the plasma *tends* to increase.

This produces the shift of HCl to the cells. At the same time there is the reduction of the oxyhemoglobin which alone would produce a more alkaline reaction within the cells. The two effects are normally so well balanced that there is practically no alteration in the [H+] of the blood.

TABLE III.

Chloride Bicarbonate Equilibrium.

Experiment No	Treatment of whole blood	Plasma chloride		Plasma bicarbonate		Increase of base determined by	
		Concentration found	Concentration expected	Concentration found.	Concentration expected	Concentration loss in chloride	Concentration gain in $NaHCO_3$.
	CO_2 per cent	*mol*	*mol*	*mol*	*mol*	*mol*	*mol*
1	5	0.0332		0.0072			
	10	0.0327	0.0336	0.0081	0.0073	0.0009	0.0008
	20	0.0316	0.0344	0.0098	0.0075	0.0028	0.0023
2	5	0.0287		0.0112			
	20	0.0280	0.0292	0.0126	0.0114	0.0012	0.0012
3	3	0.0366		0.0097			
	15	0.0340	0.0370	0.0131	0.0098	0.0030	0.0033
4	3	0.1112		0.0274			
	10	0.1081	0.1136	0.0311	0.0280	0.0055	0.0031
	20	0.1063	0.1141	0.0352	0.0281	0.0078	0.0071
5	3	0.1090		0.0294			
	10	0.1071	0.1098	0.0318	0.0296	0.0027	0.0022
	20	0.1043	0.1107	0.0366	0.0298	0.0064	0.0068
	100*	0.0977	0.1139	0.0444	0.0307	0.0162	0.0137
6	3	0.1068		0.0293			
	10	0.1043	0.1071	0.0317	0.0294	0.0028	0.0023
	20	0.1030	0.1079	0.0345	0.0296	0.0049	0.0049
	100*	0.0965	0.1119	0.0431	0.0307	0.0154	0.0124
7	3	0.1076		0.0270			
	14	0.1020	0.1098	0.0351	0.0276	0.0078	0.0075
	21	0.0998	0.1111	0.0380	0.0279	0.0113	0.0101
	37*	0.0976	0.1131	0.0408	0.0284	0.0155	0.0124
	80*	0.0960	0.1135	0.0431	0.0285	0.0175	0.0146

* Oxygen was considerably less than 20 per cent.

In the lungs the reverse process occurs; namely, a loss of carbon dioxide from the plasma, oxygenation of the hemoglobin, and a shift of hydrochloric acid back to the plasma.

Our experiments were undertaken with the hope of being able to clear up the transfer of anions and cations back and forth across the cell membrane. In Table III we have grouped together our experimental data on the loss of chloride and gain of bicarbonate in the serum. Over what might be called a very extreme physiological range (3 to 15 per cent CO_2) we find that the loss of serum chloride adequately accounts for the gain in bicarbonate in experiments conducted at room temperature (25°C.) When much higher percentages of carbon dioxide (50 to 100 per cent) were used this equivalence was not found. The loss of chloride invariably exceeded the gain in bicarbonate. We have no explanation to offer for this circumstance. The important feature to us is the equivalence over physiological ranges of carbon dioxide tensions.

In the one experiment in which the gas mixtures were analyzed our data agree with those of Hasselbalch and Warburg (1918) rather than with those of Haggard and Henderson (1920). We find that the shift of chloride with the accompanying increase of bicarbonate continues beyond 280 mm. of CO_2. Variable factors of temperature and species should be mentioned. These changes do occur to a greater extent for a given increase of CO_2 at the lower than at the higher tensions.

In Table IV we have placed our results on the shift of phosphate, sodium, and potassium. With the possible exception of phosphate, our data indicate an impermeability of the cell membrane to these ions under our experimental conditions.

We do not mean to give the impression that the corpuscles are impermeable under all circumstances. Such a condition seems to be rather improbable. However, we do think that migration of sodium and potassium plays no part in the transport of carbon dioxide. The permeability of cells to both phosphates and sulfates has not been settled. Our data with respect to the former are not conclusive.

In view of some recent comments on the ash of plasma (Mellanby and Thomas, 1920) and the possibility of sodium proteinates we are grouping some of our data in Table V to show the excess of cations over anions.

TABLE IV.

Effect of Various Carbon Dioxide Tensions on the Migration of Sodium, Potassium, and Phosphorus.

Experiment No	Treatment of whole blood.	Na per 100 cc.		K per 100 cc.		P per 100 cc.	
		Found	Expected.	Found.	Expected	Found	Expected.
	CO_2 per cent	*mg.*	*mg*	*mg*	*mg.*	*mg.*	*mg.*
2	5	249				78.9	
	20	253	253			80.5	80.3
3	3	150					
	15	151	152				
4	3	336		20.7		7.4	
	10	336	340			7.4	7.5
	20	350	345	21.6	21.2	7.3	7.6
5	3	327		21.1		8.3	
	10	331	329	21.1	21.2	8.3	8.3
	20	332	332	21.1	21.4	8.3	8.4
	100*	345	341	23.8	22.0	8.3	8.7
6	3	322		20.9		7.9	
	10	319	323	21.3	21.0	7.8	7.9
	20	326	326	21.3	21.1	7.8	8.0
	100*	345	337	22.9	21.9	7.8	8.3
7	3	339		24.4		8.7	
	14	347	346				
	21	355	350			8.6	9.0
	37*	365	356	25.7	25.7	8.6	9.1
	80*	355	358	26.3	25.8		

* Oxygen less than 20 per cent.

TABLE V.

Molecular Concentration of Anions and Cations in Serum of Beef Blood in Equilibrium with 3 Per Cent Carbon Dioxide.

Experiment No.	Na	K	Sum of the cations	Chloride.	Bicarbonate.	Phosphate.	Sum of the anions.	Excess of cations.
4	0.1461	0.0053	0.1514	0.1112	0.0275	0.0024	0.1411	0.0103
5	0.1422	0.0054	0.1476	0.1090	0.0294	0.0027	.0.1411	0.0065
6	0.1401	0.0053	0.1454	0.1068	0.0293	0.0025	0.1386	0.0068
7	0.1474	0.0062	0.1536	0.1076	0.0270	0.0028	0.1374	0.0162

Although we did not determine the sulfate, calcium, or magnesium of plasma, we venture to tabulate our figures for the other known inorganic cations and anions. De Boer (1917), considers the normal $SO_4^=$ value to be about 20 mg. per 100 cc. which amounts to a molar concentration of 0.0021. Mean values for: calcium = 0.0027 M; magnesium = 0.0010 M. Normally, then, we have a considerable excess of cations which presumably in accord with L. J. Henderson (1908) and others are combined with the proteins. We have unaccounted for, except in this way, about 7 per cent of the bases of serum.

It is evident from the table that the chloride concentration is equal to about 75 per cent of that of sodium. We have found in our analysis of human blood that a marked deviation of chloride from the normal is accompanied by a corresponding change of sodium.

CONCLUSIONS.

In vitro experiments on beef blood equilibrated with various tensions of CO_2 show the following points: (1) Equivalence of loss of chloride to gain in bicarbonate of serum. Though a migration of phosphate may occur, it is quantitatively of little importance in the transport of carbon dioxide; (2) non-transference of either sodium or potassium from cells to serum; and (3) a marked increase of corpuscle volume with increasing tensions of carbon dioxide.

PROTOCOLS.

Under the column headed, CO_2, we mean only the amount of carbon dioxide that was present in the 6 liters of gas passed through the saturators. The actual per cent of CO_2 in the saturator did not nearly equal that of the entering gas. Unless otherwise stated this mixture contained between 20 and 21 per cent of oxygen. In Experiment 7 the amount of CO_2 in the saturators was determined.

In order to diminish discrepancies due to analytical errors, artificial serums were employed in Experiments 1, 2, and 3. We had hoped to make the molar concentration of bicarbonate equal that of the chloride. This was unsuccessful. These data are included because of the close approximation of decrease of chloride to increase of bicarbonate.

The data in the protocols are used for the preparation of the tables. In each case the necessary corrections are applied as in the example given in Table II.

Experiment 1.—Defibrinated beef blood centrifuged and serum removed. An artificial isotonic serum, containing protein, glucose, potassium chloride, and bicarbonate was added. Equilibrated at 25°C. Considerable hemolysis in artificial serum. Serum bicarbonate by the Van Slyke-Cullen method.

Treatment of whole blood.	Corpuscles	NaCl		$NaHCO_3$ concentration
		Per 100 cc.	Concentration.	
CO_2 per cent	*per cent*	*mg.*	*mol*	*mol*
5	55.1	194	0.0332	0.0072
10	55.6	191	0.0327	0.0081
20	56.7	185	0.0316	0.0098

Experiment 2.—Defibrinated beef blood centrifuged and serum removed. An artificial serum, containing serum proteins, glucose, and disodium phosphate was added. Equilibration at 25°C. Plasma bicarbonate by the Van Slyke-Cullen method.

Phosphorus determination gravimetrically: the weighing of $MgNH_4PO_4 \cdot 6H_2O$ as described by Jones (1916) being used.

Treatment of whole blood.	Corpuscles	NaCl		$NaHCO_3$ concentration	Na per 100 cc	P	
		Per 100 cc.	Concentration			Per 100 cc	Concentration
CO_2 per cent	*per cent*	*mg.*	*mol*	*mol*	*mg.*	*mg*	*mol*
5	49.1	168	0.0287	0.0112	249	78.9	0.0254
20	50.0	164	0.0280	0.0126	253	80.5	0.0259

Experiment 3.—Defibrinated sheep blood. Whole blood centrifuged and serum removed. The same volume of an artificial isotonic serum, containing the serum proteins, glucose, and inorganic salts was added. The concentration of the sodium salts was made small in order to detect any part that its transfer might play in the attainment of equilibrium. Equilibration at 25°C. Considerable hemolysis occurred. Plasma bicarbonate by the Van Slyke-Cullen method.

Treatment of whole blood.	Corpuscles.	NaCl		$NaHCO_3$ concentration	Na per 100 cc.
		Per 100 cc	Concentration.		
CO_2 per cent	*per cent*	*mg.*	*mol*	*mol*	*mg.*
3	56.3	214	0.0366	0.0097	150
15	56.8	199	0.0340	0.0131	151

Experiment 4.—Defibrinated beef blood. Equilibration at 25°C. No hemolysis. Bicarbonate by titration.

Treatment of whole blood.	Corpuscles	NaCl		$NaHCO_3$ concentration	Na per 100 cc	K per 100 cc	P per 100 cc
		Per 100 cc	Concentration				
CO_2 per cent	*per cent*	*mg*	*mol*	*mol*	*mg*	*mg.*	*mg*
3	38.4	650	0.1112	0.0274	336	20.7	7.4
10	39.7	632	0.1081	0.0311	336		7.4
20	40.0	622	0.1063	0.0352	350	21.6	7.3

The volume change of corpuscles at 10 per cent CO_2 seemed to us peculiarly large in comparison with the 20 per cent. Upon the assumption that there is no exchange of nitrogen, we ran Kjeldahl determinations on the serum. The figures found follow.

CO_2 *per cent*	N per 100 cc *per cent*
3	1.017
10	1.030

When these results were used to correct the chloride and bicarbonate figures we obtained the following results.

Gain in base by loss of HCl, 0.1127 − 0.1081 = 0.0046 M

" " " as $NaHCO_3$, 0.0311 − 0.0278 = 0.0033 M

The figures from our usual method of calculation are: by loss of HCl, − 0.0055 M; by gain of $NaHCO_3$, + 0.0031 M.

Experiment 5.—Defibrinated beef blood. No hemolysis. Bicarbonate by titration.

Treatment of whole blood.	Corpuscles	NaCl		$NaHCO_3$ concentration	Na per 100 cc	K per 100 cc	P per 100 cc.
		Per 100 cc.	Concentration				
CO_2 per cent	*per cent*	*mg*	*mol*	*mol*	*mg*	*mg.*	*mg*
3	40.9	637	0.1090	0.0294	327	21.1	8.3
10	41.2	626	0.1071	0.0318	331	21.1	8.3
20	41.8	610	0.1043	0.0366	332	21.1	8.3
100	43.4	571	0.0977	0.0444	345	23.8	8.3

Experiment 7.—Defibrinated beef blood. No hemolysis. Bicarbonate by titration. Equilibrated with the following gas mixtures.

CO_2 *per cent*	O_2 *per cent*
3	20–21
14	20–21
21	20–21
37	13.0
80	4.0

Treatment of whole blood.	Corpuscles	NaCl		$NaHCO_3$ concentration.	Na per 100 cc.	K per 100 cc.	P per 100 cc.
		Per 100 cc.	Concentration.				
CO_2 per cent	*per cent*	*mg.*	*mol*	*mol*	*mg.*	*mg.*	*mg.*
3	46.2	629	0.1076	0.0270	339	24.4	8.7
14	47.3	596	0.1020	0.0351	347		
21	47.9	584	0.0998	0.0380	355		8.6
37	48.8	570	0.0976	0.0408	365	25.7	8.6
80	49.0	561	0.0960	0.0431	355	26.3	

Addendum.

We are indebted to Dr. Donald D. Van Slyke for calling our attention to a point in our paper which perhaps needs further explanation. The point concerns the fact that as stated on page 380 we have determined the shift of ions occasioned by different tensions of CO_2, but in all cases have determined the bicarbonate by titration to an arbitrarily fixed and *constant* pH and have thus excluded from consideration the additional shift of base from serum proteins to carbonic acid which doubtless occurs with *changing* pH.

With Dr. Van Slyke's consent we append his comment on this point, with which we agree.

"There are two types of reactions with plasma by which increase in $[H_2CO_3]$ causes increase in $[BHCO_3]$; *viz.*,

$$H_2CO_3 + BCl = BHCO_3 + HCl \qquad (1)$$

the HCl being transported into the blood cells; and

$$H_2CO_3 + B\ \text{Protein} = BHCO_3 + H\ \text{Protein} \qquad (2)$$

(Van Slyke, 1921). Of these two reactions, the titration to the constant end-point measures only the first. The results therefore indicate that the above chloride reaction accounts for practically all the bicarbonate change due to migration of ions; *i.e.*, that Cl^- is the only ion other than HCO_3^- that plays a significant part in the acid-base shift between cells and plasma. The results do not indicate the relationship between the amount of $BHCO_3$ formed as the result of this migration and the amount formed by reactions with plasma buffers of the type exemplified above by Reaction (2)."

BIBLIOGRAPHY.

Barcroft, J., The respiratory function of the blood, Cambridge. 1914, 58.

Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

de Boer, S., *J. Physiol.*, 1917, li, 211.

Collip, J. B., *J. Biol. Chem.*, 1921, xlvi, 61.

Doisy, E. A., and Bell, R. D., *J. Biol. Chem.*, 1921, xlv, 313.

Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.

Gürber, A., *Jahresb. Tierchem.*, 1895, xxv, 164.

Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xlv, 189.

Hamburger, H. J., *Wien. med. Woch.*, 1916, lvi, 519, in this paper the author refers to earlier papers on this subject.

Hasselbalch, K. A., and Warburg, E. J., *Biochem. Z.*, 1918, lxxxvi, 410.

Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 169.

Henderson, L. J., *J. Biol. Chem.*, 1921, xlvi, 411.

Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920, liv, 129.

Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.

McLean, F. C., Murray, H. A., Jr., and Henderson, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 180.

McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

Mellanby, J., and Thomas, C. J., *J. Physiol.*, 1920, liv, 178.

Smith, M., *J. Biol. Chem.*, 1921, xlv, 437.

Smith, L. W., Means, J. H., and Woodwell, M. N., *J. Biol. Chem.*, 1921, xlv, 245.

Van Slyke, D. D., *Physiol. Reviews*, 1921, i, 141.

Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.

EXPERIMENTAL RICKETS IN RATS.

II. THE FAILURE OF RATS TO DEVELOP RICKETS ON A DIET DEFICIENT IN VITAMINE A.*

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PLATES 1 TO 3.

(Received for publication, May 28, 1921.)

The most recent hypothesis regarding the etiology of infantile rickets places it in the group of deficiency diseases resulting from a lack of vitamine. This hypothesis was advanced from a theoretical standpoint by Funk, and later was sustained by Mellanby as the result of experiments on dogs (1). The latter believes he has proved that when puppies are deprived of the so called fat-soluble vitamine (vitamine A) they develop rickets. The conclusions of Mellanby were accepted by the committee on accessory food factors of Great Britain (2) appointed by the Medical Research Committee and the Lister Institute.[1] This report considers the fat-soluble factor, or a factor with similar distribution, synonymous with the antirachitic factor, and presents a table of antirachitic foods of three grades of potency. In a previous paper one of the authors has shown that infants do not develop rickets when on a diet containing a minimal amount of this factor, but that on the contrary they not infrequently develop rickets in spite of receiving food rich in this vitamine, and concluded, therefore, that this unidentified factor could not be regarded as the antirachitic vitamine, and that it does not exert a controlling influence on the development of this disorder (3). More recently McCollum, Park, and their associates (4) have stated that in their experience rats fed on certain diets deficient in the so called fat-soluble A or in both that substance and calcium develop "a condition identical with the rickets of human beings." In a paper just published (5) they express the opinion that the fat-soluble A vitamine is not the sole cause of rickets, but that it cannot be excluded as an etiological factor in the production of this disorder.

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* Read in abstract before the Society of Experimental Biology and Medicine, May 18, 1921.

[1] Experimental evidence against the contention of Mellanby that the fat-soluble vitamine is the determining factor in rickets, has been presented by Paton, Findlay, and Watson (*Brit. Med. J.*, 1918, ii, 625), and by Paton and Watson (*Brit. J. Exp. Path.*, 1921, ii, 75).

The following investigation concerns itself with the effect on young rats of a diet markedly deficient in the fat-soluble vitamine. That lesions similar to those of infantile rickets can be induced in young rats has been shown recently by the work of Shipley and his associates (5) and by Sherman and Pappenheimer (6). For our purpose rats weighing about 30 gm. were placed on a basal ration (Diet C) similar to that employed by Drummond and Coward (7):

Casein	21 per cent
Rice starch	57 " "
Salt mixture	5 " "
Crisco	17 " "
Yeast	60 mg.

The casein was extracted with about twice its volume of cold alcohol by means of slow filtration which lasted 3 to 4 hours, until the last washings came through colorless. It was then extracted with ether (using about twice the volume of casein) for about 48 hours, and employing two changes of ether. The salt mixture was that used by Osborne and Mendel. Yeast was the Harris extract, prepared according to the formula of Osborne and Wakeman (8). One group of animals was also given orange juice, 0.5 cc. daily. Both the yeast and the orange juice were not incorporated in the food, but given separately, so as to make certain that they were consumed in full amount. In addition to this ration, which was complete except for an almost total lack of the fat-soluble vitamine, we employed Diet B, which was complete in all respects, 6 per cent of butter fat having replaced an equivalent amount of Crisco. In some instances Diet B was given practically throughout the experimental period, in order to allow of a comparison of growth and health between

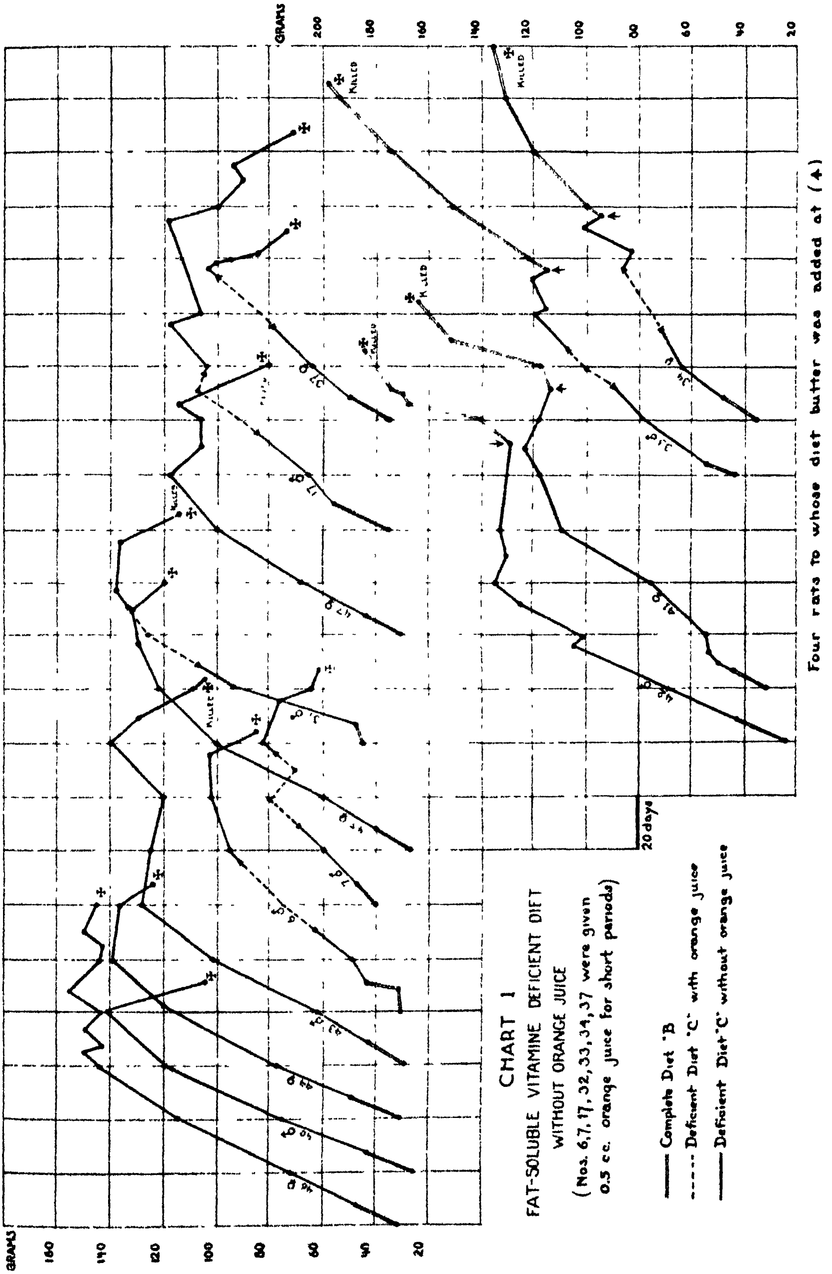

CHART 1

FAT-SOLUBLE VITAMINE DEFICIENT DIET WITHOUT ORANGE JUICE

(Nos. 6, 7, 17, 32, 33, 34, 37 were given 0.5 cc. orange juice for short periods)

—— Complete Diet 'B'

- - - - Deficient Diet 'C' with orange juice

—— Deficient Diet 'C' without orange juice

Four rats to whose diet butter was added at (↓)

a complete ration and one deficient in the fat-soluble vitamine (Chart 3); in other instances Diet B was substituted where the deficient diet had been maintained for months (Chart 1).

The accompanying charts and tables illustrate our results. It will be seen that the rats on this deficient diet failed to gain in weight after a period of about 2 months, that somewhat later they began to lose in weight, and that they invariably died prematurely. This is clearly illustrated by the graphs of Charts 1 and 2. The corresponding tables (Tables I and II) show that these rats in almost all instances showed lesions of keratitis, which is considered distinctive of this vitamine deficiency. These are the two criteria which are accepted as pathognomonic of a lack of the fat-soluble factor, growth failure and keratitis, so that this diet must be judged to have been markedly lacking in this essential factor. That such was the case is evident from a survey of Chart 3, which differs from the preceding groups (Charts 1 and 2) merely in that butter was added to the dietary. Here growth invariably was excellent. This distinction is well exemplified also in Chart 1 and Table IV, which show the sharp rise in the growth curve and the rapid cure of the ophthalmia when 6 per cent of butter was substituted for an equal amount of Crisco.

One group of rats on the deficient dietary was given 0.5 cc. of orange juice daily, whereas the other group received no antiscorbutic foodstuff. These groups were constituted in order to determine whether a lack of antiscorbutic still further retarded growth and favored the development of the eye lesions. As is well known, opinion is divided as to whether rats require antiscorbutic vitamine or whether they thrive normally when deprived of this factor. On adding orange juice a definite and prompt increase in weight was brought about in some instances, but this gain did not persist for long periods. In general, it may be stated that the curves in Chart 2, representing rats which received orange juice do not appear superior to those in Chart 1, where this antiscorbutic was lacking. Ophthalmia occurred less frequently and with less severity among the rats which received orange juice. This was more noticeable clinically than on pathological examination; among the eleven rats which received no orange juice, only one failed to develop ophthalmia during life (Table I),

TABLE I.

Fat-Soluble Vitamine Deficient Diet, No Orange Juice.

Serial No.	Days on deficient diet.	Eyes during life.	Infections during life.	X-ray.	Pathological examination.		
					Rickets.	Ophthalmia	Other lesions.
6	113	Purulent exudate and puffiness.		Negative 111th day.	None.*	Slight keratitis.*	Normal.
7	80	Purulent exudate.	Diarrhea.		" *	No keratitis.*	Hemorrhagic colitis; cestode infection.
17	139	" "			" *	Minimal lesion.*	Focal abscesses in kidney. Diphtheritic gastritis.*
32	77	" " and puffiness.			" *	Corneal ulcer; keratitis.*	Acute tracheitis.*
37	65	Clear.	Diarrhea.		" *	None.*	Intestines contain thin fluid and gas.
40	63	Puffiness.	Bloody urine.		" *	Conjunctivitis.*	Pyelitis (L); bronchopneumonia.*
43	138	Purulent exudate; puffiness.	Diarrhea.	Negative 137th day.	" *	Mild keratitis.	Suppurative infection of lung and submaxillary gland. Intestinal cestodes.
44	82	Bloody exudate.			" *	Minimal lesions.*	Pyelitis (L).
45	96	" "			" *	Conjunctivitis.	" (double).
46	116	" " puffiness.	Purulent urine.		" *	Very slight keratitis.*	
47	90	Bloody exudate.			" *	No keratitis.*	None.

* Confirmed by microscopic examination.

TABLE II.

Fat-Soluble Vitamine Deficient Diet, with Orange Juice.

Serial No	Days on deficient diet.	Eyes during life.	Infections during life.	Pathological examination.		
				Rickets	Ophthalmia	Other lesions.
2	119	Slight exudate.	Ulcer under jaw.	None.*	None.	Healing ulcer in neck.
3	115	Clear.	Infected ear; rhinitis.	" *	Slight keratitis.*	Parasitic cysts in renal pelvis.
5	151	Exudate and puffiness.		" *	" opacity of cornea.	Suppurative pneumonia.*
9	119	Clear.	Diarrhea; rhinitis.	" *	Slight keratitis.*	None.*
11	91	"	Otitis media.	" *	" " *	Purulent otitis (L).
12	154	Purulent exudate and puffiness.	Diarrhea.	"	" " *	Submaxillary abscess; abscesses of lung.
13	105	Clear.	Rhinitis.	" *	No keratitis.	Parasitic infection of renal pelvis.
16	169	Purulent exudate and puffiness.		" *	Slight keratitis.*	None.
21	115	Clear.		" *	" " *	Bronchiectasis.*
22†	182	Purulent exudate; puffiness.				
27	108	Bloody exudate.		None.*	" infiltration of cornea.*	None.
28	59	Clear.	Diarrhea.	" *		
31	53	Bloody exudate.		" *	Conjunctivitis.	Intestinal cestodes.*
36	54	Clear.	Otitis media.	" *	Very slight keratitis.*	Focal necroses in liver.*

* Confirmed by microscopic examination.

† X-ray, negative, 182nd day. Not killed.

TABLE

Long Period on Deficient Diet, Followed by Addition of Butter, with Orange Juice.

Serial No.	Total days on diet.	Period on deficient diet.	Eyes during life.		Infections during life.	Pathological examination.		
			While on deficient diet.	While receiving butter		Rickets	Ophthalmia	Other lesions.
		days						
8	210	172	Clear.	Clear.	Diarrhea.	None.	None.	None.
14	202	169	Bloody exudate one day, (145th).	"	"	"*	"	Intestinal cestodes.
24	202	169	Purulent exudate on 144th to 169th day.	Cured in 4 days.	Pus in urine.	"	"	Vesical calculi.
33	145	74	Clear.	Clear.	Diarrhea.	"	"	
34	145	70	"	"	"	"	"	
41	135	104	Bloody exudate 97th to 104th day.	Cured in 3 days.		"	"	None.
42	135	104	Bloody exudate and puffiness, 95th to 104th day.	" " 2 "		"	"	"

whereas among the fourteen which did receive an addition of orange juice, in seven the eyes were at all times normal (Table II).

In this connection it should be borne in mind that some preliminary observations of Osborne and Mendel (9) indicated that orange juice contains traces of the fat-soluble vitamine.

Probably a deficiency of the antiscorbutic factor intensifies the alteration of the cells brought about by other nutritive deficiencies. In experiments on scurvy in guinea pigs ophthalmia and keratitis have been observed by us. Similar lesions have been reported in the course of human scurvy (10). In this connection it may be mentioned that conjunctivitis, unaccompanied by puffiness of the lids, occurs not infrequently among white rats, especially where they have not been kept in the dark.

TABLE IV.

Control Rats on Full Diet for Entire, or Almost Entire, Period.

Serial No	Total days on diets	Period on deficient diet	Eyes during life.	Infections during life	Pathological examination.		
					Rickets	Ophthalmia.	Other lesions.
			With orange juice.				
15	114	11th to 33rd day.	Clear.		None.	None.	None.
18	148	0	“	Rhinitis.	“	“	“
20	148	0	“		“	“	“
25	179	0	“		“ *	“ *	“
26	131	6th to 30th day.	“		“	“	“
38	114	0	“		“	“ *	“
48	137	0	“		?*	“ *	“
			Without orange juice.				
1	121	11th to 44th day.	Slight exudate on 89th day.		None.	None.	None.
10	117	11th to 44th day.	Clear.		“	“	“
35	114	6th to 42nd day.	“		“	“	“

* Confirmed by microscopic examination.

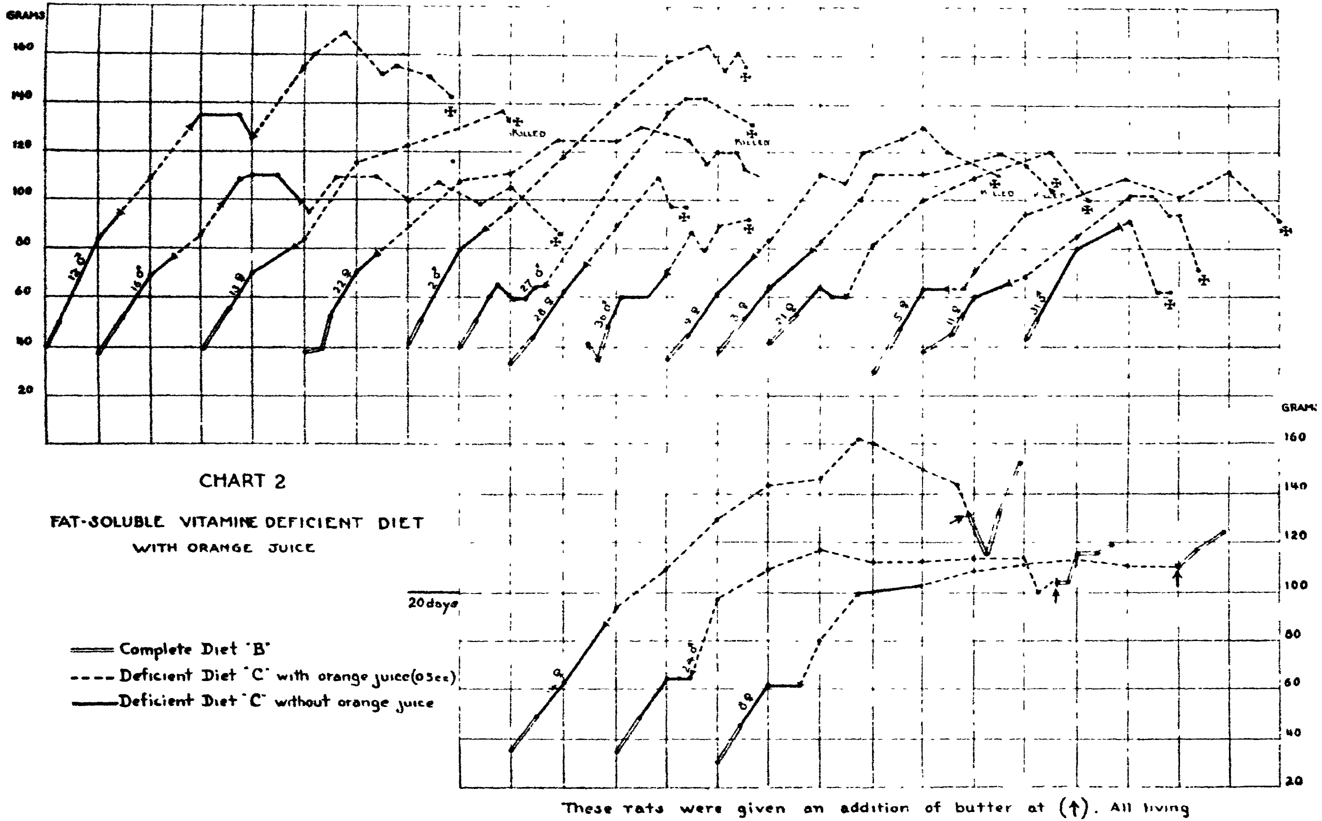

CHART 2

FAT-SOLUBLE VITAMINE DEFICIENT DIET
WITH ORANGE JUICE

These rats were given an addition of butter at (↑). All living

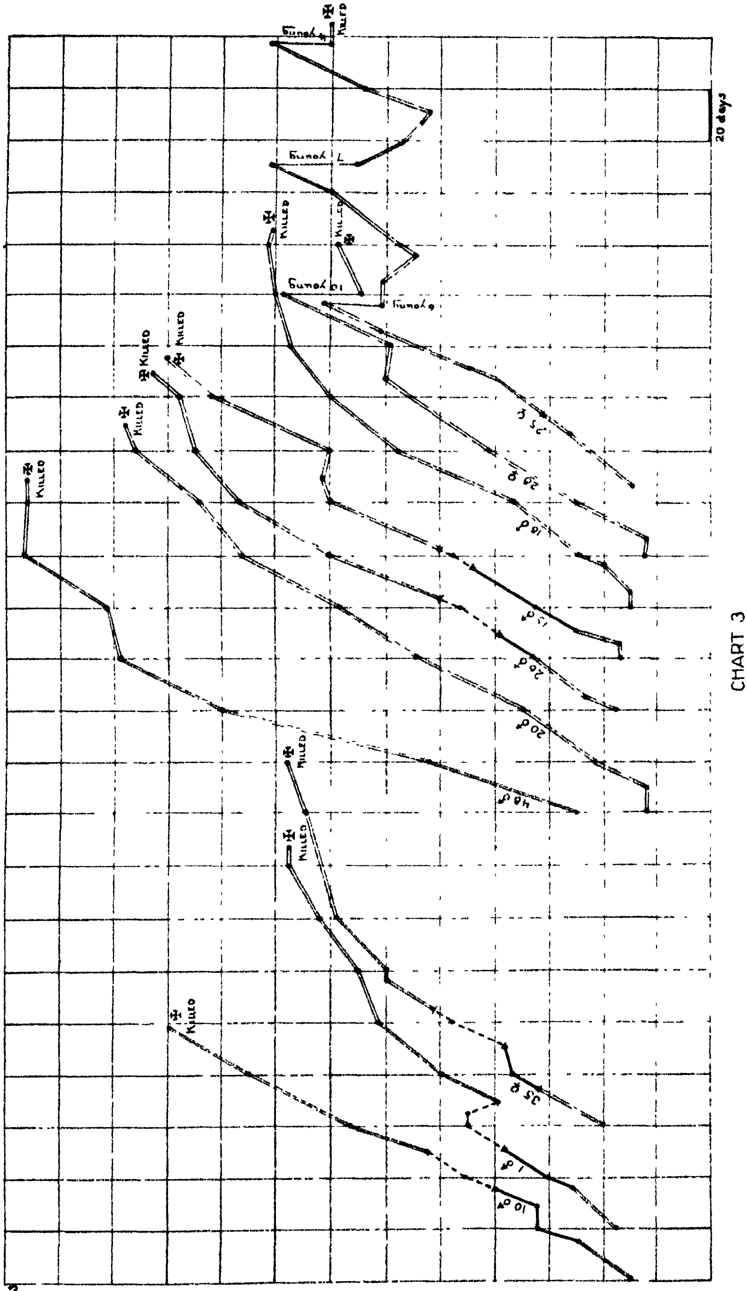

CHART 3
FAT-SOLUBLE VITAMINE ADEQUATE DIET "B"

Pathological Examination.

As regards gross changes the skeletal system was found normal in all respects. In no instances were there the deformation of the thorax, the beading and angulation of the costochondral junctions, the multiple infractions surrounded by masses of callus, which form so striking a feature of rickets in rats. The long bones also gave evidence of no undue pliability, curvature, or epiphyseal enlargement. Under the microscope,[2] however, certain deviations from the normal picture were found quite regularly. As will be evident from the accompanying description and illustrations, these lesions were in no way suggestive of rickets but were correlated rather with the deficient growth of the animals.

The zone of proliferating cartilage was narrow; the columns rarely exceeded four cells in depth, and frequently were composed of not more than two or three cells. The matrix of the cartilage in this zone of proliferation was invariably densely calcified, not only the capsules of the cartilage cells but the tissue between them taking a deep purplish blue stain indicative of calcium. This was confirmed in some of the preparations by applying von Kossa's silver nitrate method.

The most striking departure from the normal was found in the subchondral zone. Instead of a series of stout trabeculæ showing an orderly parallel arrangement about the calcified cartilage matrix, and directed in the long axis of the rib, the primary spongiosa was defective. In many cases it was represented by two or three delicate and irregularly disposed trabeculæ leaving the calcified cartilage exposed over a large portion of the chondrocostal line; in others there was a continuous thin transverse plate of fully calcified bone, limiting the cartilage and fusing with the calcified matrix. The osteoblasts, surrounding these trabeculæ or lying against the subchondral bone plate, were inconspicuous in contrast to their swollen appearance in actively proliferating bone. Osteoid tissue was conspicuously absent here as also along the completely calcified cortical layer.

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[2] After fixation in Muller formol, ribs were decalcified for 5 to 15 days in Muller's fluid, embedded in paraffin and stained with hematoxylin eosin. The examination of the long bones has not yet been completed.

The trabeculæ of the secondary spongiosa were often better developed, having been formed during the period of more active growth. The marrow usually was of the normal cellular type.

TABLE V.

Histological Picture of Costochondral Junctions of Rats Fed Complete and Incomplete Diets.

	Complete diet.	Fat-soluble A deficient diet	Rachitic diet.
Zone of proliferating cartilage.	2–4 cells deep. Regular arrangement. Matrix calcified.	1–4 cells deep. Columns often slightly separated. Matrix calcified.	1–20 cells deep. Irregular prolongations into metaphysis. Matrix uncalcified.
Primary spongiosa.	Stout trabeculæ corresponding to columns of cartilage cells, parallel alignment.	Trabeculæ reduced in number and size, irregular in arrangement, often fused into thin plate, completely calcified.	Composed chiefly of osteoid, with core of calcified cartilage or bone. Dense tissue with narrow vascular spaces.
Secondary spongiosa.	Well developed.	Well developed in some cases, deficient in others.	Dense trabeculæ with wide osteoid margins.
Cortex.	Narrow osteoid margin in young growing rats; invisible in older animals.	No visible osteoid margin. No infractions.	Irregular broad subperiosteal and endosteal osteoid margins. Frequent infractions, with masses of cartilaginous and osteoid callus.

In some rats which had acute suppurative infections in other tissues (submaxillary gland, kidneys), there was an unusual proportion of polymorphonuclear leucocytes among the marrow elements. In a few preparations the marrow immediately about the cartilage was depleted of blood-forming elements, and composed predominantly of pale polyhedral cells, often forming multinucleated masses about the cartilage.

The features which differentiate the bones of these animals maintained on fat-soluble vitamine deficient diet from those of rats on an adequate diet and pursuing normal growth as well as well as from those with rachitic lesions, are brought out in Table V.

From the foregoing comparison it is clear that the skeletal changes in rats on a fat-soluble deficient diet are in no respect suggestive of rickets, but may be interpreted as due to an inactive osteogenesis. This is what might be expected in view of the stationary or declining weight and arrested growth.

The ocular lesions have been so thoroughly studied by Stephenson and Clark (11), and by Wason (12) that detailed reference need not be made to our experiences. The histological changes were such as have been described by these observers. Of the 25 rats comprised in Tables I and II it will be noted that in 5 the corneæ were normal on microscopic examination.

Incidental infections were encountered in a large proportion of the rats; notably, submaxillary abscesses, suppurative infections of the urinary tract, and bronchiectases. Infestation with the cestode, *Hymenolepis murina,* was also extremely common. Vesical calculi, described by Osborne and Mendel (13) in rats deprived of the fat-soluble vitamine, were encountered at autopsy in one instance (Rat 24). In this animal it was associated with pyelitis. Its occurrence in this connection is probably due in part to the susceptibility to infections induced by a diet lacking in this vitamine.

CONCLUSION.

Young rats receiving a diet complete except for a lack of the fat-soluble vitamine invariably failed to grow and generally developed keratitis. The keratitis developed less frequently when the ration included orange juice. If this diet is continued for a period of months the animals die, either of inanition or, more often, of some intercurrent infection. The skeletons of such rats show no gross changes whatsoever. Microscopic examination of the bones of 22 rats on a ration of this character presented definite signs of a lack of active osteogenesis, but in no instance lesions resembling rickets. In view of these results and their conformity with our previous experience in regard to infantile rickets, we are of the opinion that this vitamine cannot be regarded as the antirachitic vitamine, and that, if the diet is otherwise adequate, its deficiency does not bring about rickets.

BIBLIOGRAPHY.

1. Mellanby, E., *Lancet*, 1919, i, 407.
2. Hopkins, F. G., and Chick, H., *Lancet*, 1919, ii, 28.
3. Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.
4. Shipley, P. G., Park, E. A., McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1921, xlv, 343.
5. Shipley, P. G., Park, E. A., McCollum, E. V., and Simmonds, N., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 160.
6. Sherman, H. C., and Pappenheimer, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 193.
7. Drummond, J. C., and Coward, K. H., *Biochem. J.*, 1920, xiv, 661.
8. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.
9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xlii, 465.
10. Zlocisti, T., *Klin. Monatsbl. Augenh.*, 1917, lix, 572.
11. Stephenson, M., and Clark, A. B., *Biochem. J.*, 1920, xiv, 502.
12. Wason, I. M., *J. Am. Med. Assn.*, 1921, lxxvi, 908.
13. Osborne, T. B., and Mendel, L. B , *J. Am. Med. Assn* , 1917, lxix, 32.

EXPLANATION OF PLATES.

Plate 1.

Fig. 1. Normal rib. Rat 82. Weight 158 gm. 52 days on complete diet; continuous growth and gain in weight. Decalcified 3 days in Muller's fluid.

Fig. 2. Rib of Rat 17. 139 days on fat-soluble A deficient diet. Maximum weight 118 gm. Terminal weight 71 gm. Decalcified 10 days in Muller's fluid.

Zone of preparatory calcification is very narrow, not exceeding three cells in depth. Cartilage cells separated by densely calcified plate. Primary spongiosa represented by continuous bony plate, without definite trabeculæ. Cortex thick, without visible osteoid margin. Osteoblasts inconspicuous.

PLATE 2.

FIG. 3. Rib of Rat 44. 82 days on fat-soluble A deficient diet. Maximum weight 140 gm. Terminal weight 125 gm. Decalcified 5 days in Muller's fluid.

Zone of preparatory calcification not over four cells in depth, regular, matrix completely calcified. Osteogenesis at epiphysis is fairly active. Spongiosa composed of coarse trabeculæ, with absent or inconspicuous osteoid margin. Cortex broad and completely calcified. Marrow cellular, not fibrous.

FIG. 4. Rib of Rat 58. 34 days on rickets-producing diet. Marked rachitic lesions. Note increased width and irregularity of proliferative cartilage, absence of calcium deposition, great excess of osteoid in region of metaphysis and about cortex. Decalcified 5 days in Muller's fluid.

PLATE 3.

FIG. 5. Rat 16. 175 days on fat-soluble A deficient diet. X-ray. Absence of rachitic lesions at upper epiphysis of tibia.

FIG. 6. Rat 59. 41 days on rickets-producing diet. X-ray, showing rachitic changes in upper epiphysis of tibia.

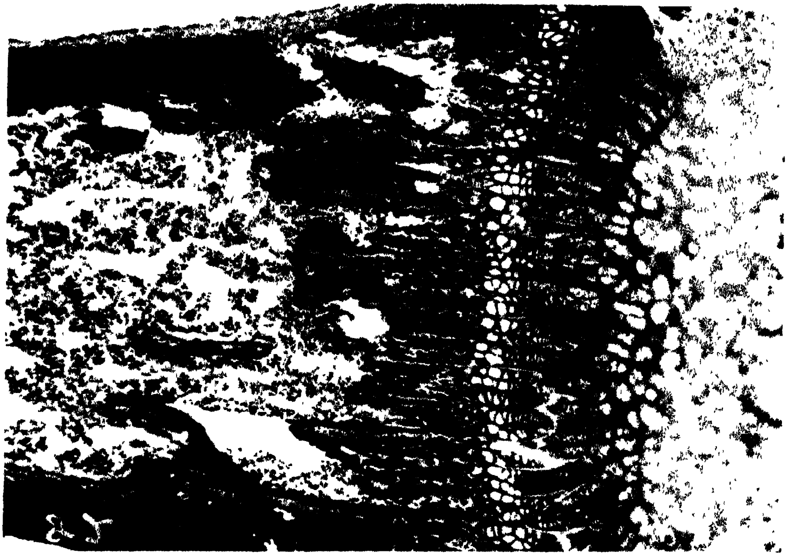

FIG. 1.

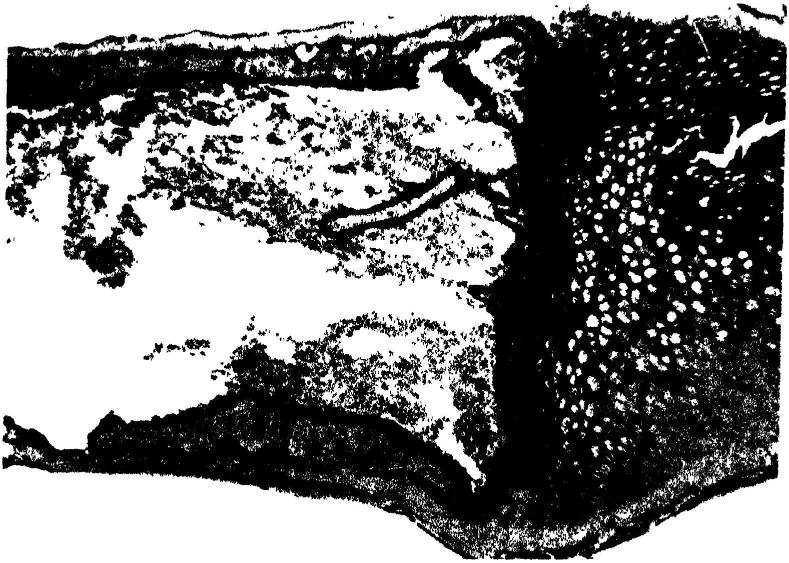

FIG. 2.

(Hess, McCann, and Pappenheimer: Experimental rickets in rats. II.)

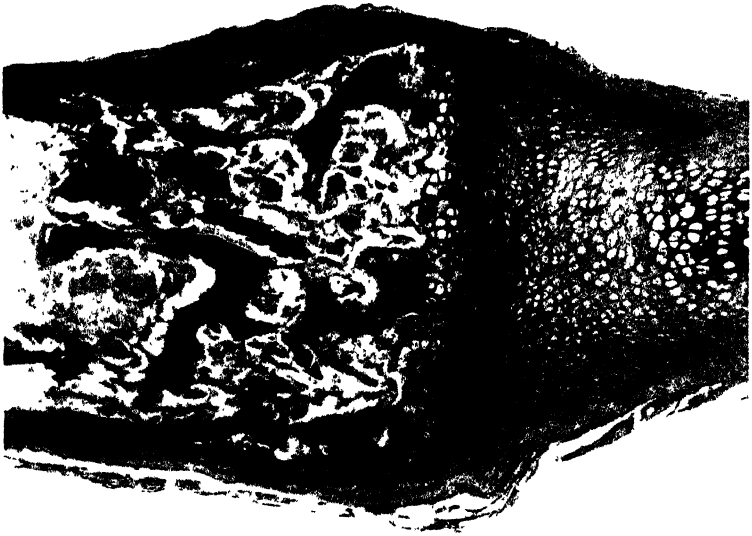

Fig. 3.

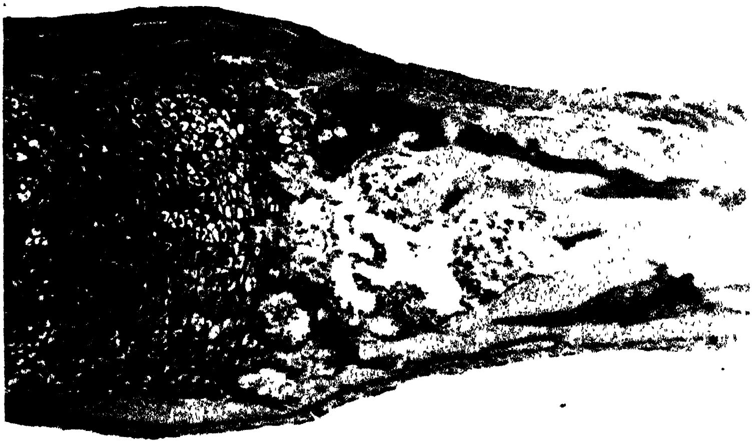

Fig. 4.

(Hess, McCann, and Pappenheimer: Experimental rickets in rats. II.)

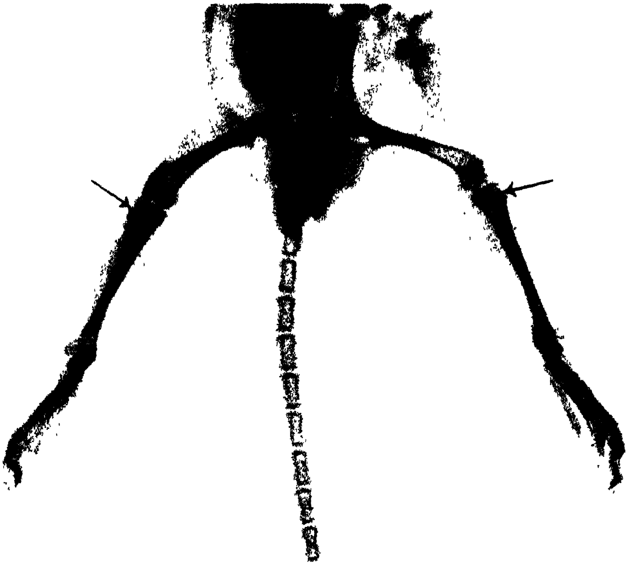

Fig. 5.

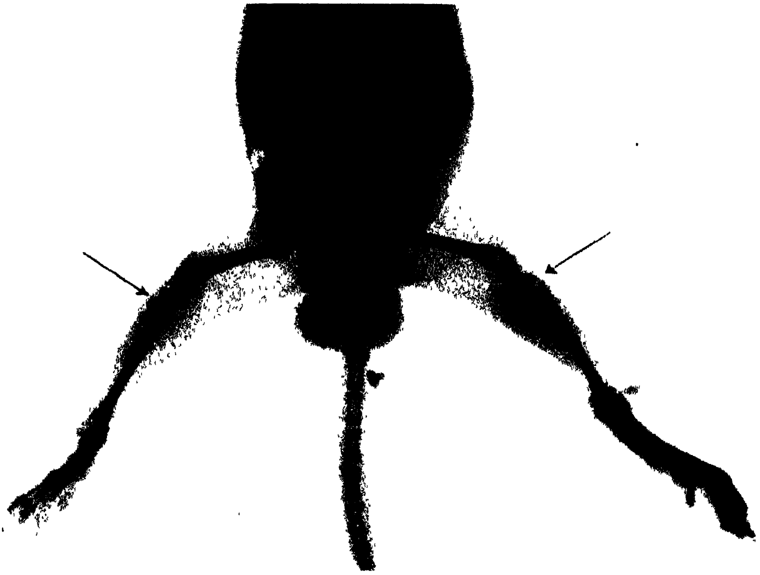

Fig. 6.

(Hess, McCann, and Pappenheimer: Experimental rickets in rats. II.)

METHODS OF EXTRACTING AND CONCENTRATING VITAMINES A, B, AND C, TOGETHER WITH AN APPARATUS FOR REDUCING MILK, FRUIT JUICES, AND OTHER FLUIDS TO A POWDER WITHOUT DESTRUCTION OF VITAMINES.

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(From the Laboratory of Physiological Chemistry, the University of Minnesota Medical School, Minneapolis.)

(Received for publication, May 16, 1921.)

I.

As the population increases, the question of food supply becomes more urgent, and the vitamines being necessary food constituents need to be conserved. It is therefore important to develop methods for extracting them from plants that are not considered edible and for preserving them in foodstuffs that require storage. Except for the perfection of the drying apparatus, the methods here described, were worked out in 1919–20. Methods A, B, and C, are especially devised for the vitamines, respectively A, B, and C. Each product is, in reality, a mixture of vitamines with one predominating. By these methods the vitamines are extracted with very little loss, and concentrated without the use of very expensive reagents.

The unique features of the methods are believed to be; first, the extraction of vitamine A from green leaves or fruit skins by the use of high pressure (after moistening with alcohol). Second, the separation of the resinous and lipoid material from the water-soluble portion in extracting vitamine B by increasing the H ion concentration up to the isoelectric point of these colloids; and third, the removal of the sugars from the B and C extracts by fermentation with baker's yeast. All processes are carried out in the absence of oxygen, and the drying is done very quickly.

In making the A extract, green leaves or fruit skins may be used. These are dried, preferably in the absence of oxygen, and then ground to a powder. This powder is moistened with alcohol by being thrown into a container half filled with boiling 95 per cent alcohol and allowed to remain without application of heat for 24 hours. The mass is then placed in a strong canvas bag and subjected to a pressure of 5,000 pounds to the square inch in a suitable press. The press-cake may be ground in a mill and reextracted in the same manner. The press-juice is dried in the apparatus described below. If, however, it is desired to recover the solvent, a preliminary concentration may be done in a vacuum pan. The product may be a sticky powder when absolutely dry, but absorbs water from the air, and becomes pasty. It contains resinous or fatty substances. It is of a waxy consistency when made from spinach and absolutely dry, but is hygroscopic.

The preparation of the water-soluble B is made from wheat germ or other foodstuff, such as yeast, which is rich in this vitamine. The wheat germ is treated in the same manner as the green leaves up to the pressing out of the extract, except that 80 per cent alcohol is used. The press-cake is ground in a mill and extracted again in the same manner. The press-juices are concentrated in a vacuum pan to about one-tenth of the original volume or until the first precipitate starts to form. An equal volume of water is now added and hydrochloric acid is added slowly and with stirring until a bulky and sticky precipitate forms. This is filtered off and the precipitate washed with distilled water and the washings are added to the filtrate. The precipitate is dried and extracted with benzene in any convenient extraction apparatus and the benzene is evaporated. The residue contains some of the wheat oil. The filtrate is brought to $pH = 4$ to 5 and is fermented until the reducing sugar is lowered to about 1 per cent of its original value. The yeast is filtered off and added to the next batch of wheat germ and the filtrate is evaporated to dryness. In both the filtrate and the benzene extract, the water-soluble B is in quite concentrated form and there is practically no loss except the portion absorbed by the yeast and which would be recovered in the next batch.

The water-soluble C is extracted from fruits or tomatoes. If oranges are used they are pressed and the juice is run into vessels previously filled with carbon dioxide so as to exclude the oxygen of the air, and baker's yeast added and a cover kept on so that no air enters. The fermentation is allowed to proceed at room temperature until the reducing sugar is lowered to about 1 per cent of its original value which requires about 24 to 48 hours. The juice is then filtered with the exclusion of air and is condensed by spraying (in the apparatus described below) in the absence of oxygen, until the volume is reduced to about one-twentieth of the original volume. It is then thrown into four volumes or more of 95 per cent alcohol and the precipitate thus formed is filtered off and the filtrate sprayed and reduced to dryness with the same precautions.

These three preparations may be designated A, B, and C. Preparation A contains sufficient fat-soluble A vitamine so that about 0.05 to 0.1 gm. daily added to a ration free from fat-soluble A will produce normal growth in a rat. It also contains considerable water-soluble B.

Preparation B is very rich in water-soluble B vitamine so that a very small dose of either the lipoid or water-soluble fraction will cure a pigeon of polyneuritis. The lipoid fraction will revive the hypodynamic heart of the turtle, but the water-soluble fraction is ineffective. Both fractions are effective growth stimulants.

Preparation C contains sufficient antiscorbutic vitamine so that about 5 to 10 cg. a day will prevent scurvy in a guinea pig on a basic diet supposedly free from water-soluble C for a period of about 2 months.

Preparation C was in one instance purified further by the removal of citric acid, but since it is highly hygroscopic and deteriorates more rapidly (when moist) when not acid, further work on this preparation was postponed awaiting the perfection of the drying apparatus.

The tests for the concentration of vitamines were made on rats, guinea pigs, and pigeons. The rats and guinea pigs were kept in metabolism cages, one unit of a 10 compartment cage being shown in Fig. 1. In case metabolic data were not kept, the pan and beaker under the cage were removed and a newspaper placed under the cage to catch the excreta. Drinking water was always present in the bottle. The basic diet for the guinea pigs was equal parts of white flour and alfalfa meal, and for the rats was 10 per cent vitamine-free casein, 6 per cent dehydrated sea water, and 84 per cent white flour. In case vitamine A was investigated the rat diet contained 5 per cent dry Fleischmann's yeast and in case vitamine B was investigated the rat diet contained 1 gm. of butter fat per day.[1]

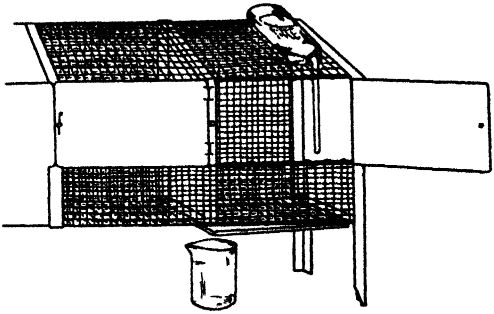

Fig. 1.

II.

The Drying Apparatus.

Many forms of drying apparatus were tried, such as evaporation at low temperature in a rotating drum by a blast of air, vacuum pans through which a small stream of carbon dioxide and nitrogen passed, atomizer sprays in flue-gas, and evaporation in front of an electric fan at room temperature. None of these methods was efficient, and, therefore, the following method was devised.

The apparatus, Fig. 2, consists of a chamber near or at the bottom of which is the flue-gas exhaust, and in the center of the ceiling of which is the flue-gas and spray intake. The spray or distributor is rotated on the same shaft with the fan propelling the flue-gas at a speed of 5,000 revolutions a minute by an electric motor. The milk or other liquid passes down from a tank through a cock which regulates its flow into the hollow shaft of this apparatus. The furnace is built on top of the chamber.

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[1] Some of the tests were made by Dr. M. A. Shillington, Helen Brenton, H. D. Reineke, and E. Dunlap, and these workers may publish details of their tests elsewhere.

It is constructed of fire-brick plastered with fire-clay, and has two flues that may be closed with valves, one extending upward, and the other closed by nichrome wire gauze and extending downward through an opening in the center of the ceilng. The apparatus propelling the flue-gas and making the spray is shown in detail in Fig. 3. It revolves on a steel tube that serves as a shaft and which is driven by a pulley at the top. Inside this steel tube is an aluminum or brass tube, which touches the steel tube only at the extreme top and bottom. Between the steel tube and the aluminum tube is an air space to prevent the passage of heat

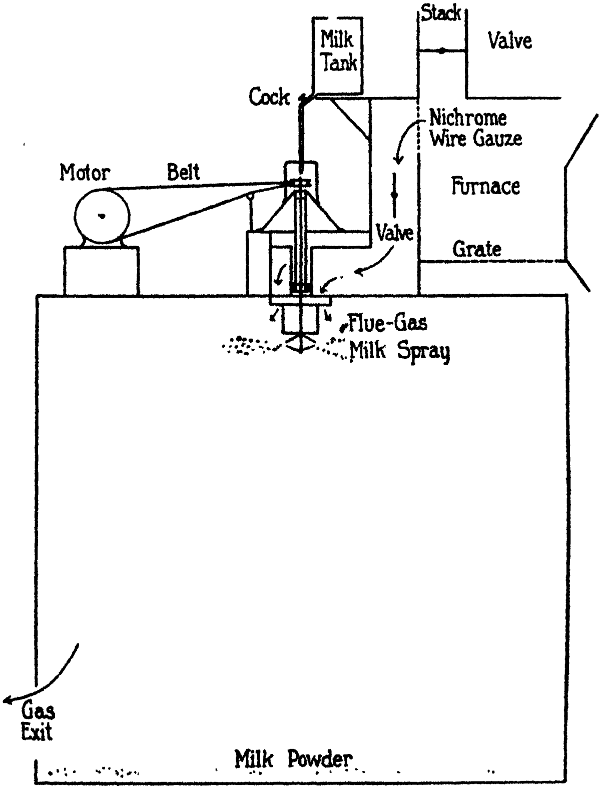

FIG. 2.

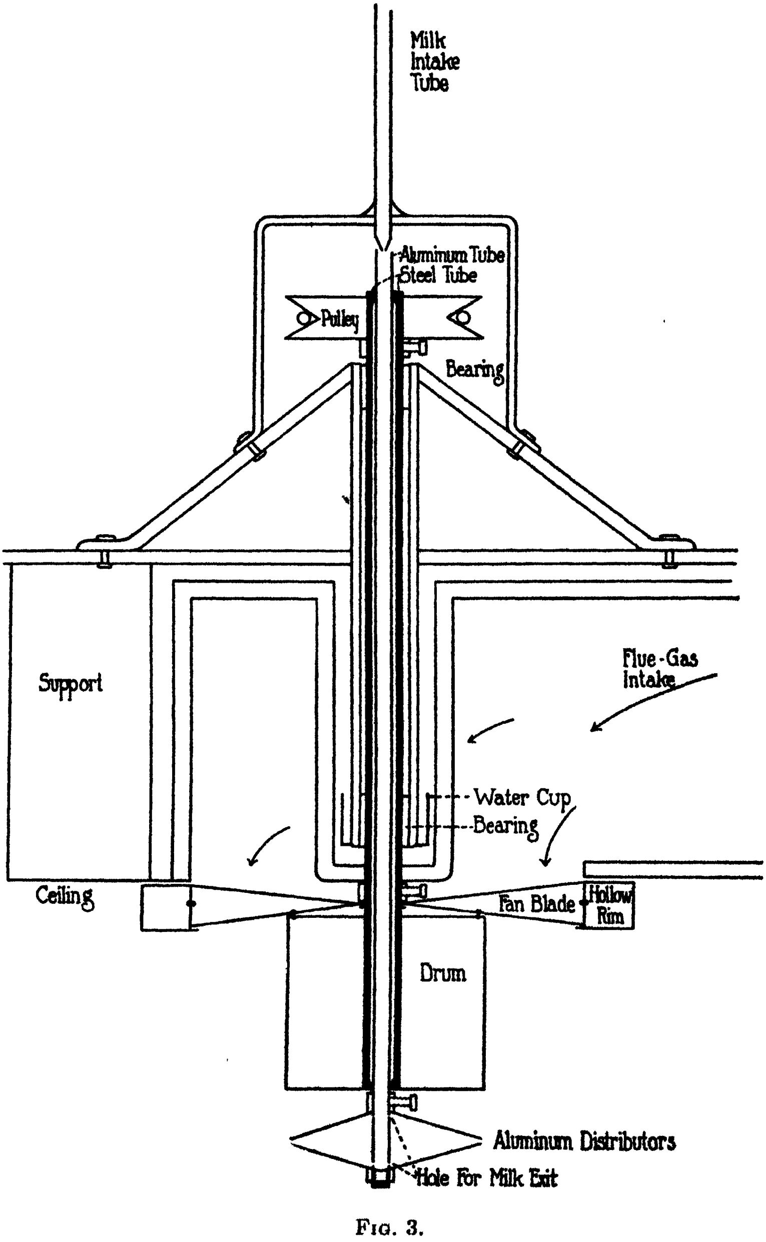
Milk Intake Tube
Aluminum Tube
Steel Tube
Pulley
Bearing
Support
Flue-Gas Intake
Water Cup
Bearing
Ceiling
Fan Blade
Hollow Rim
Drum
Aluminum Distributors
Hole For Milk Exit

FIG. 3.

into the milk or other liquid being sprayed. Above the aluminum tube is fixed a nozzle which spurts a stream of the milk into the aluminum tube. The milk passes down the aluminum tube by gravity and out through a series of holes bored through the aluminum tube near the bottom, whence it strikes the surface of two cone-shaped aluminum distributors and spreads out on their surfaces and is thrown off from the periphery, which is cut into numerous saw-tooth-like points. From each point drops of liquid are thrown off by the rotation of the apparatus, producing a fine spray. On the steel tube, just above the aluminum distributors is a hollow drum which serves to prevent eddy currents and retard passage of heat to the steel tube. Above the drum is a fan with a hollow rim. This fan forces the flue-gas downward, cutting the spray at right angles and rapidly evaporating the water. In order to prevent undue heating of bearings in which the steel tube rotates, an air space is provided between the flue-gas intake and the stationary tube in the ends of which the bearings are located. Also a deep groove or "cup" filled with water is provided on the lower end of this stationary tube. The aluminum distributor and fan are shown in perspective in Fig. 4. The hollow rim of the fan is useful to prevent burning of some of the spray that might be drawn against the rim of the fan by eddy currents. The portion of the flue right above the

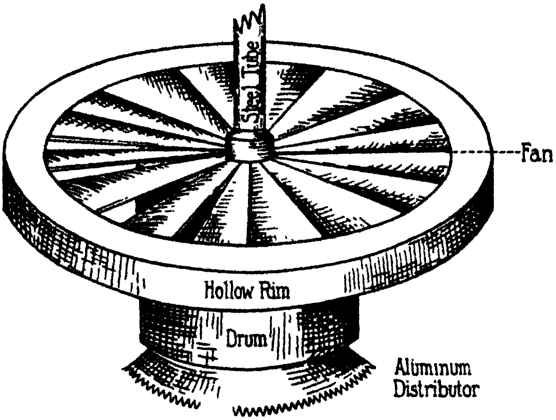

FIG. 4.

fan is made of wire screen plastered on both sides with asbestos furnace cement. In order to prevent the presence of oxygen in the flue-gas the amount of air must be reduced to a point at which some carbon monoxide persists. This carbon monoxide will form a crust of carbonyl on metallic surfaces, which will peel off and drop in the chamber. It is, therefore, best to have all metal coming in contact with the heated flue-gas made of nichrome, which seems more resistant than other metals or alloys, and the amount of metal should be reduced as much as possible. Therefore, all stationary metal parts exposed to flue-gas are covered with asbestos furnace cement. In operating the apparatus the furnace is filled with fairly large pieces of charcoal or coke, and this is ignited by means of illuminating gas. The valve in the flue or stack that extends upward is opened until the heat of the furnace is sufficient to drive off volatile substances in the fuel. The valve in the stack is then closed and the valve in the flue extending downward into the chamber is opened and the motor is set going, thus driving the hot flue-gas into the chamber. When the chamber is heated to about 150° at the top a little water is passed downward through the aluminum tube in order to cool it. Then the fluid from the tank is allowed to pass slowly into the aluminum tube. The rate of flow of this fluid is regulated by the cock so that the temperature at the bottom of the chamber remains at about 80°. The temperature is greater at the top than at the bottom, and, if the walls are cooled, a condensation of moisture will take place within the chamber. It is therefore desirable to have the walls of non-conducting material, or else have the space surrounding these walls filled with the exhaust gas. It is desirable to have a series of thermometers with bulbs extending through the wall into the chamber at intervals from the top to the bottom. By reading these thermometers the flow of the fluid can be so regulated that the spray will always be reduced to a powder at the lowest possible temperature. The incoming flue-gas may be about 1,000°, but is instantly cooled by coming in contact with the spray. It is necessary, however, for the temperature within the chamber to be high enough to prevent recondensation of the moisture evaporated. Just what temperature that will be depends on the exact temperature of the flue-gas before meeting the spray and on whether there is any loss of heat through the walls or floor of the chamber. The temperature must, in any case, be higher than that required by warm-air-drying systems, but this disadvantage seems to be compensated by the greater stability of the vitamine in the absence of oxygen. In case milk is sprayed the absence of oxygen prevents the oxidation of the fat.

As to how well this apparatus will work on a very large scale needs to be determined, but as an experimental apparatus, it has the great advantage that the cost of installation is comparatively small and changes are easily made. The necessity of steam boilers, steam radiators, and condensers is obviated. The greatest loss is the heat that passes out in driving off the volatile constituents of the fuel before starting the spray, but this heat could be used for other purposes. Also the exhaust gas, if not saturated with moisture may be used for drying vitamine-containing products on tray dryers. So far this apparatus has been used for drying the vitamine extracts described above and also for drying orange juice and milk.

The dimensions of this apparatus as it now stands are as follows: The chamber is 8 feet square and 12 feet high, the aluminum distributor is 3 inches in diameter, and the fan 6 inches in diameter inside the rim. The flue is also 6 inches in diameter. A $\frac{1}{4}$ horse power motor is used. The size of the motor could be greatly decreased by the use of ball bearings replacing the Babbit metal bearings in which the steel tube rotates. The main difficulty experienced has been the burning of some of the spray around the edge of the flue-gas intake. Practically no spray sticks to the walls if the temperature is maintained high enough. In case it is desired merely to condense the spray, there is considerable condensation of moisture on the walls, and care should be taken that this does not drip into the condensed spray which falls into enamelled pans on the floor. Very little dust in the form of ashes need pass into the chamber if the apparatus is operated properly. The fan acts as a centrifugal dust separator. Two methods have been tried to improve the dust separation. One is the passage of gas through an asbestos filter, and the other is the modification of the fan so that it forms a more efficient centrifugal dust separator. The latter method is open to the objection that the dust accumulating in the fan must be periodically removed or else it may throw the fan out of balance. The complete combustion of the fuel in the furnace is desirable. Apparently the nichrome wire gauze acts as a catalyzer when heated red hot. It is, however, necessary to have the bed of coals deep enough so that some carbon monoxide is formed in order to obviate the danger of an excess of oxygen. Apparatus for the analyses of flue-gas might be installed if the drying apparatus were used on a large scale.

While operating the apparatus as described above, the powder is kept at about 80° until the end of the operation. If it is desired to keep the powder cool, the flue-gas outlet may be made a few feet above the bottom, and the chamber below this outlet kept filled with cold CO_2 or other gas. The flue-gas escaping from the outlet may be cooled and dried and used to fill the lower portion of the chamber. If this cool dry flue-gas is warmed slightly it may be used to complete the drying of the spray in case this was not accomplished by the time the spray reached the level of the flue-gas exit. The powder may be transferred to a vacuum drier to complete the drying.

Previous condensation of milk or other liquid to be sprayed is a disadvantage and may cause it to be thrown onto the walls. Condensation increases viscosity and increases the size of the particles of powder resulting from the spray. These large powder grains dry superficially, but if then placed in a hermetically sealed container, the moisture from the inside of the grains appears on their surfaces. If such powder, soon after being formed, is placed in a vacuum desiccator, it may be completely dried without coalescence of the grains.

Addenda.—Owing to the hygroscopic nature of orange power the drying apparatus has been modified as follows: The floor of the drying chamber is made of a box 1 foot high, the top of which is made of muslin. Air dried over $CaCl_2$ is forced into the box and comes slowly through the muslin, thus completing the drying of the powder that falls on top and keeping it cool.

HEMATO-RESPIRATORY FUNCTIONS.

XII. RESPIRATION AND BLOOD ALKALI DURING CARBON MONOXIDE ASPHYXIA.

By HOWARD W. HAGGARD and YANDELL HENDERSON.

(Investigations performed for the United States Bureau of Mines in the Laboratory of Applied Physiology, Yale University, New Haven.)*

(Received for publication, May 17, 1921.)

Carbon monoxide asphyxia has long been accepted as involving the typical condition of acidosis. The decreased oxygen-carrying power of the blood was supposed to result in incomplete combustion in the tissues and a production of organic acids. The increase of lactic acid in the blood and urine (Araki (1) and Ryffel (2)) was thus explained. Without this foundation of correct observation, but incorrect inference, the theory of acidosis in a wide variety of conditions would probably never have attained the hold which it now has on current thought.

It is true, as found by Saiki and Wakayama (3), that under carbon monoxide asphyxia the blood alkali is greatly decreased. But, as we shall show, this decrease is not of acidotic origin.

In previous papers (4, 5, 6) we have demonstrated that a decrease of blood alkali may be induced in two almost diametrically opposite ways: (1) the acidotic process, and (2) the acapnial process. In the acidotic process strong acids find their way into the blood, partially neutralize the $NaHCO_3$ of the plasma, and overload the corpuscles with acid. A differential test of this condition may be carried out by causing the subject to inhale air containing 6 or 8 per cent CO_2. If the acid intoxication is extreme, and the inhalation is pushed, the animal is soon killed by the excessive acidity thus induced in the blood. A normal animal is not harmed.

In the acapnial process, on the other hand, various influences and conditions excite the respiratory center through agencies other than increase of C_H. This results in overbreathing and an excessive elimination of CO_2 which leaves the blood abnormally alkaline. A gradual compensatory disappearance of alkali from the blood follows. Subjects in this condition respond favorably to inhalations of CO_2. Such inhalations not only overcome the alkalosis, but also rapidly recall alkali to the blood. It will be seen in the experiments here to be reported that it is the acapnial, and not the acidotic, process which comes into play. In a condition of acidotic origin the administration of sodium bicarbonate should be beneficial. It should be injurious in acapnia; and, as we find, it is injurious and even fatal in carbon monoxide asphyxia.

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* Published by permission of the Director.

All the experiments were carried out upon dogs. Enough illuminating gas, containing about 25 per cent carbon monoxide, was mixed with air in a large spirometer to afford the desired concentration of carbon monoxide, namely 15 to 45 parts in 10,000 of air, that is 0.15 to 0.45 per cent. A mask was made air-tight over the animal's head with adhesive plaster. An inspiratory valve and tube led from the spirometer to the mask, and an expiratory valve from the mask to the outside air, or at intervals to a wet gas meter of low resistance. Such conditions are quite comfortable and painless. The femoral artery was exposed under cocaine, and blood samples were taken at intervals. Part of each sample was analyzed, and part was equilibrated with three tensions of CO_2 in air and analyzed, as in our previous work.

The following protocol, of which the data are given in Experiment 1 and Fig. 1, is typical. The subject breathed air containing 0.25 per cent of carbon monoxide until death resulted after 237 minutes. The volume of respiration gradually increased nearly threefold. The blood alkali, or CO_2-combining power, fell gradually, but more slowly and to a less degree than the respiration increased. Consequently, the CO_2 content of the arterial blood, as the table in the protocol shows, was relatively so much reduced that the ratio H_2CO_3: $NaHCO_3$, and presumably therefore the C_H, was constantly subnormal. There was no acidosis, but a marked alkalosis until near the end when respiration was as usual depressed.

The most complete distinction between the acidotic and acapnial processes is to be gained by plotting the results of a series of blood gas analyses in a CO_2 diagram. Such a diagram for this experiment is shown in Fig. 1. The dissociation curves are drawn from the data of the protocol and the position of the arterial blood on each curve is indicated by a dot. The abscissæ in such a diagram are proportional to the alveolar tension of CO_2 and therefore to the content of H_2CO_3 in the arterial blood. The ordinates express the combined CO_2 or blood alkali. The diagonal line OC expresses the normal C_H or pH, since for every point in this line the relation of abscissa : ordinate is constant. Thus whenever the arterial point falls to the left of the OC line the ratio H_2CO_3: $NaHCO_3$ is below normal, and overbreathing, acapnia, and alkalosis are indicated. This condition prevailed throughout this experiment until the terminal depression of breathing set in. That these conditions are induced and determined by the volume of breathing is shown by the observations noted on the curve for the respiration (Resp.) in the figure.

Experiment 1.—Dog, female, 11 kilos. From 12 o'clock on the animal breathed air containing 0.25 per cent carbon monoxide from a large gasometer through a mask and valves. Blood drawn from the femoral artery under cocaine; part analyzed for CO_2 content, remainder equilibrated with 40, 72, and 18 mm. CO_2 at body temperature and analyzed.

Curve No.	Time.	Volume of respiration per minute.	Blood equilibrated with CO_2.			Arterial blood.			CO_2 ratio	C_H7 (Char. 8).
			18 mm	40 mm	72 mm.	CO_2 content.	CO_2 dissolved	CO_2 combined.		
		liters	*vol. per cent*	*vol per cent*	*vol per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*		
1	11.45	4.5	23	38	48	38	2.8	35.3	0.79	0.63
	12.25	5.2	23	38	48	38	2.8	35.3	0.79	0.63
2	1.10	6.1	23	37	46	31	2.0	29.0	0.69	0.55
	1.40	6.3								
	2.00	7.4	18	32	42	25	1.7	23.3	0.73	0.58
	2.25	8.0								
	2.45	9.0	18	33	41	24	1.5	22.5	0.70	0.56
3	3.10	12.0	17	33	41	18	1.1	16.9	0.69	0.55
4	4.00	7.0	13	25	32	14	1.2	12.8	0.93	0.74

Gasps, apnea, death.

The data of this experiment are expressed graphically in Fig. 1 and are discussed in its legend.

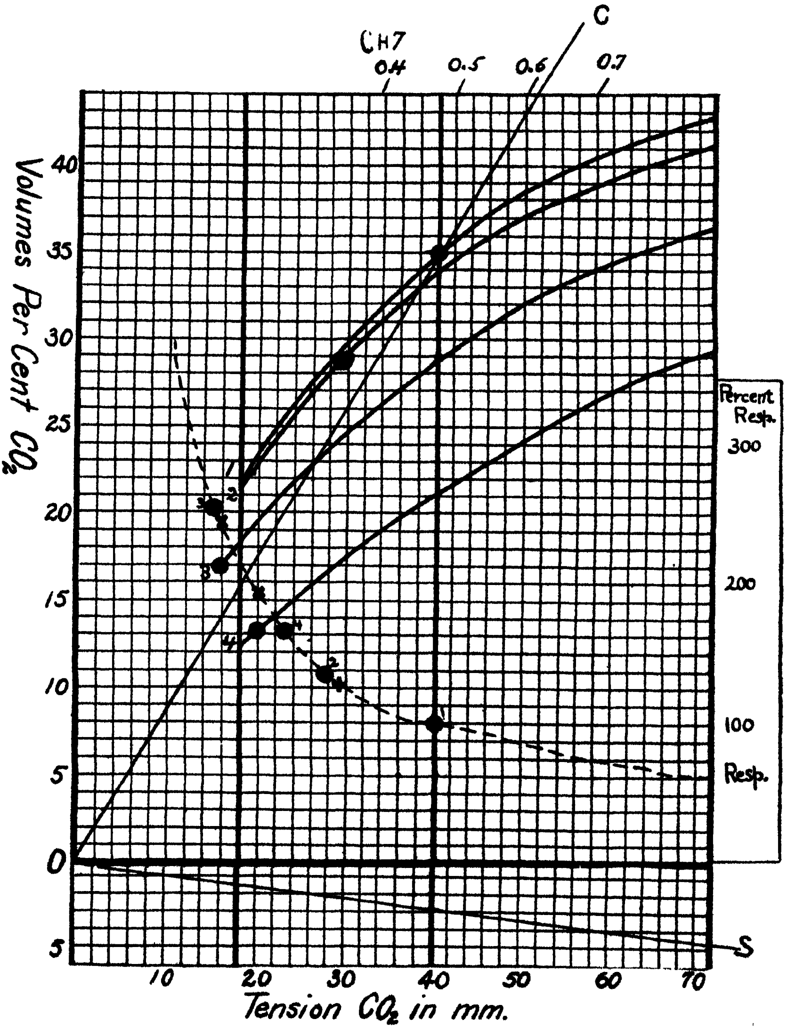

Fig. 1. The CO_2 diagram (5) plotted from the data of Experiment 1. The lowering of the dissociation curve from 1 to 4, expresses the decrease of blood alkali. The line OC expresses the normal C_H of this animal. The deviation of the arterial points (•) to the left of the OC line on Curves 2 and 3 indicates that the excessive breathing is producing acapnia and alkalosis. Only during the terminal depression of breathing does a deviation to right, or toward acidosis, occur. The volumes of breathing (Resp.) as estimated from the arterial blood and as measured directly coincide in the first 3 sets of observations but deviate slightly from agreement in the fourth (• and × on curve marked Resp.).

The protocols of several other experiments are here given in abbreviated form. In addition to the points brought out in Experiment 1, some of these experiments throw light on additional matters. Thus in Experiments 2 and 3 the animals were removed from the gas-containing atmosphere before death, and allowed to recover. During the period of recovery respiration was for a time markedly depressed. A similar depression has been observed by us previously in experiments following true acid intoxication. It causes the ratio H_2CO_3: $NaHCO_3$ to rise much above normal. Such an acidosis is, we think, to be regarded as a restorative process; it is an effort of the body to recall its alkali into use in the blood.

We are inclined to believe that acidosis, that is a spontaneous high ratio of H_2CO_3: $NaHCO_3$, or high C_H, always has this meaning. In other words alkalosis is an effort, and the normal method of the organism to lower the blood alkali and acidosis is Nature's method of calling more alkali into use (Experiments 2-5).

Experiment 2.—Dog, male, 16 kilos From 12 o'clock on the animal breathed air containing 0.17 per cent carbon monoxide. At 6 o'clock it was in coma It was then removed to fresh air, and recovered spontaneously. The blood alkali was estimated by equilibrating the blood with 40 mm. CO_2 at body temperature and analyzing for CO_2. The volume of respiration is expressed in liters per minute.

	Normal.	Breathing 0 17 carbon monoxide						Recovering
Time	11.45	12 35	12 50	2 10	3 00	4 00	6 00	8.00
Respiration	6 0	6 0	8 3	9 0	12 0	18.0	21 0	4.0
Blood alkali	41	40	36	30	32	28	26	32
Arterial CO_2	40	40	26	24	25	18	17	30

Experiment 3.—Dog, male, 11 kilos. Conditions similar to Experiment 2. Inhaled 0.16 per cent carbon monoxide from 12 to 9 o'clock. Then in coma, removed to fresh air, recovered spontaneously.

	Normal.	Breathing 0 16 carbon monoxide.						Recovering		
Time	11.45	1.15	2.15	4 10	5.10	8 00	9.00	9.30	10 50	12.00
Respiration	5.8	8.0	6.5	7 5	8.6	9.2	12.0	7.6	8.0	6.9
Blood alkali	47	47	45	43	39	36	36	45	44	44
Arterial CO_2	48	43	43	39	32	29	28	44	43	43

Experiment 4.—Dog, female, 7 kilos. Inhaled 0.49 per cent carbon monoxide from 12 o'clock on. Respiration failed at 12.40, and the heart stopped beating at 12.46.

	Normal.	Breathing 0 49 per cent carbon monoxide		Dying.	
Time	11.45	12.36	12.38	12 42	12.46
Blood alkali	49	31	31	30	29
Arterial CO_2	48	19	17	21	26

Experiment 5.—Dog, female, 7 kilos. Inhaled 0.45 per cent carbon monoxide for 44 minutes. Then 60 cc. of 2 per cent solution of sodium bicarbonate were injected intravenously. Tetany developed, respiration ceased, and death followed.

	Normal.	Breathing 0 45 per cent carbon monoxide.	Dying after $NaHCO_3$.
Time	11.45	12.43	12.46
Blood alkali	50	38	75
Arterial CO_2	51	20	52

Note that in these experiments the arterial CO_2 leads the blood alkali downward. Thus augmented breathing induces acapnia and alkalosis.

In Experiment 5 an intravenous injection of a moderate amount of $NaHCO_3$ caused death in respiratory failure and tetany. This experiment affords an instructive contrast to some other experiments recently published by one of us (7) in which it was found that when a dog remains in an atmosphere of carbon monoxide until death the presence of CO_2 (7 per cent) distinctly prolongs life. By tending to neutralize the alkalosis, the CO_2 prevents the occurence of apnea vera, due to excessive loss of CO_2, which is the common mode of death in carbon monoxide asphyxia.

Oxygen Consumption and Respiratory Quotient under Carbon Monoxide Asphyxia.

It might be supposed, if the current theory of asphyxial, or rather anoxemic, acidosis were correct, that during carbon monoxide poisoning the oxygen consumption by the body as a whole would begin to fall (owing to lactic and other organic acids escaping oxidation) coincidentally with overbreathing. In Experiments 6 and 7 this is seen not to be the case. The respiratory quotient rises greatly, but the oxygen consumption, instead of falling, actually increases in one experiment almost to the end, due probably to the muscular exertion of the respiratory movements.

Experiment 6.—Dog, male, 7 kilos. The oxygen metabolism and respiratory quotient were obtained by placing the dog in an air-tight glass chamber of 300 liters capacity (minus a number of liters equal to the weight of the dog in kilos). An electric fan circulated the air in the chamber and a spray of water on the top of the chamber kept the temperature at 22°C. At intervals gas samples were drawn from the chamber and analyzed for oxygen and CO_2. The decrease of oxygen (1.7 per cent per hour) and increase of CO_2 were sufficient for calculation of the metabolism and respiratory quotient, but insufficient to affect the condition of the animal appreciably.

After obtaining the normal figures for oxygen consumption, CO_2 output, and respiratory quotient, fresh air plus carbon monoxide to the desired concentration were run into the chamber and the measurements continued until death.

Time	Remarks	Air in chamber.		O_2 consumption per minute	Respiratory quotient
		O_2	CO_2		
min.		*per cent*	*per cent*	*cc.*	
0	Dog in chamber.	20.91	0.04		
15	Quiet.	20.50	0.41	74.5	0.80
30	"	20.08	0.73	76.0	0.76
47	Moving	19.58	1.13	90.4	0.79
60	Quiet.	19.18	1.44	73.5	0 77

The chamber was then thoroughly ventilated with fresh air, closed again, and a charge of 1,450 cc. of pure carbon monoxide was run in.

0	Gas run in.	20.83	0.05		
15	Quiet.	20.43	0.35	72.6	0.75
30	"	19.94	0.77	89.0	0.86
39	Slightly excited.	19.37	1.31	103.5	0.95
45	Occasional gasps.	19.14	1.69	104.4	1.60
52	Frequent "	19.01	1.74	50.6	1.10
53	Respiration failed.				

Experiment 7.—Dog, male, 14 kilos. Conditions as in Experiment 6.

Time.	Remarks	Air in chamber		O₂ consumption per minute	Respiratory quotient.
		O_2	CO_2		
min		*per cent*	*per cent*	*cc.*	
0	Dog in chamber.	20.90	0.05		
30	Dog quiet.	19.75	0.98	110	0.76
45	" "	19.15	1.41	112	0.79

The chamber was then thoroughly ventilated with fresh air, closed again, and 1,500 cc. of pure carbon monoxide were run in.

Time.	Remarks	O_2	CO_2	O₂ consumption per minute	Respiratory quotient.
0	Gas run in.	20.85	0.06		
30	Dog quiet.	19.64	0.96	106	0.81
45	Dyspnea.	18.94	1.56	118	0.96
62	"	18.31	2.76	102	1.90
69	Respiration failed.				

Overbreathing and Acapnia as the Cause of Low Blood Alkali.

It will be seen that the foregoing observations afford no support whatever to the idea that the lactic acid formed in the body during carbon monoxide asphyxia is the cause of the hyperpnea, or of the decrease of blood alkali. On the contrary the data are in complete accord with the view expressed in previous papers from this laboratory, and with the conclusions reached by Haldane and his collaborators (8) that asphyxia causes not acidosis but alkalosis. Macleod (9) suggests the view, which we had put forward (6, 10) that the lactic acid which is formed in such conditions is to be regarded, not as causing acidosis, but rather as assisting in neutralizing the alkalosis. Evidently carbon monoxide asphyxia induces overbreathing and a considerable degree of acapnia, a conclusion supported by the ill effects of injection of alkali in Experiment 5. The beneficial effects of inhalation of 6 to 10 per cent CO_2 in dogs after carbon monoxide asphyxia have recently been described by us (11). With Coburn (12) we have demonstrated the same beneficial effects on men in the depression following prolonged etherizations, in which condition also acapnia is a factor.

The view now receiving general assent, that asphyxia may cause overbreathing and acapnia, is decidedly at variance with the theory that the C_H of the blood is the sole chemical factor in the control of breathing. Evidently the oxygen supply is also a factor. But merely to say that "oxygen deficiency stimulates respiration" leaves wide open the question as to how the deficiency acts. We need some substance, chemical process, or physiological mechanism to take the place heretofore erroneously assigned to lactic acid as a respiratory stimulant. It must however be a substance (if it is a substance at all) which does not act through an acid property, but as a respiratory stimulant of another order. There are such substances, but for the most part they have not been demonstrated to occur in the body. Ethyl ether in small amounts is a powerful respiratory excitant (13), and in unskillful or prolonged anesthesia may cause profound acapnia. Hydrogen sulfide, as we have found in experiments as yet unpublished, is even more potent. Indeed, inhalation of minute amounts of H_2S has in our hands proved capable of inducing the most intense hyperpnea, and subsequent fatal apnea vera. That this is not an effect of H_2S as an acid is demonstrated by the fact that intravenous injections of Na_2S are equally effective. We have recently been engaged in trying to identify in the blood some sulfur compound readily altered by oxygen, which might play the rôle of the hypothetical respiratory stimulant which we have called "respiratory X." As yet, however, our results in this search are inconclusive. The problem is clear only on one point: Oxygen deficiency does not stimulate respiration through the formation of a substance with acid properties.

Carbon Monoxide Asphyxia after Section of the Vagi.

It seems well established, or at least accepted, that CO_2 and C_H influence respiration through action on the respiratory center itself (14). It occurred to us that perhaps oxygen deficiency, or anoxemia, stimulates not the center itself, but rather the vagus endings in the lungs. The probabilities are, of course, strongly against this hypothesis (which we are now investigating, however), and we mention it now only because it has led to an observation which is of critical importance here. Thus among other tests of this idea, we have carried out experiments on the influence of carbon monoxide asphyxia upon respiration and blood alkali in dogs in which the vagi were cut.

The striking result of this operation (Experiments 8 and 9) is that the hyperpnea and dyspnea which are ordinarily seen under carbon monoxide asphyxia (Experiments 1 to 7) are entirely lacking after section of the vagi; and the blood alkali remains practically unaltered throughout the whole course of asphyxia, and even up to death. It is not necessary in the present connection to assign to the vagus section, or to consider here, any effect beyond the prevention of excessive breathing. This prevents the loss of CO_2 (acapnia) and the usual compensatory fall

Experiment 8.—Dog, male, 16 kilos. Under local anesthesia (cocaine) the trachea was exposed, cannulated, and connected with double valves, so that the expired air passed into a recording spirometer. Blood samples were obtained from the femoral artery. After the normal volume of respiration in liters per minute, and the arterial CO_2 had been determined, the vagi were cut, and the minute volume of respiration was again determined. Then the inspiratory valve was connected to a large spirometer containing 0.5 per cent carbon monoxide in air, which the animal inhaled until death occurred 50 minutes later. The hemoglobin of the blood was then found to be combined with carbon monoxide to the extent of 76 per cent.

	Normal	Vagi cut	Breathing 0.5 per cent CO					
Time, *min.*	0	15	25	35	45	55	65	75
Respiration, *liters*	3.1	3.6	3.4	3.2	3.5	3.4	2.8	0
Arterial CO_2	48		46				48	

Note that in this and the following experiment, in marked contrast to all the preceding experiments, there was no hyperpnea and no fall of blood alkali.

Experiment 9.—Dog, male, 12 kilos. Conditions similar to Experiment 8. At death HbCO = 72 per cent.

	Normal.	Vagi cut		Breathing 0.5 per cent CO.				
Time, *min.*	0	20	25	38	45	60	70	80
Respiration, *liters*	2.6	4.1	3.2	2.9	3.7	2.8	2.6	0
Arterial CO_2	40		·				38	Death.

of blood alkali. If the acidotic process were involved in asphyxia the blood alkali would fall as well without hyperpnea as with it. Evidently therefore, oxygen deficiency as such does not cause a production of acid in the tissues, or at least, no passage of such acids into the blood, for the blood alkali is not at all or only slightly decreased.

This demonstration is so clear-cut a disproof of the theory of anoxemic acidosis under carbon monoxide as to bring into serious doubt the possibility of such an acidosis under any condition. The papers from this laboratory (15) a few years ago, and from other laboratories more recently in which the reasoning has been based upon the assumption that such a condition may occur, will require fundamental reconsideration. Evidently the increase of acidity in tissues to which the circulation is obstructed is wholly due to accumulation of H_2CO_3 (16), and not at all to neutralization of alkali by lactic and other strong acids. When an increase of lactic acid, or rather lactate, occurs it indicates as above shown, not acidosis, but alkalosis.

CONCLUSIONS.

Carbon monoxide asphyxia induces, not acidosis, but alkalosis. The lowering of blood alkali is due to the acapnial, not the acidotic, process. The anoxemia induces excessive breathing (up to 300 per cent or more), and the decrease of blood alkali is an attempt at compensation.

The rate of oxygen consumption is scarcely, if at all, decreased until death is imminent, but the respiratory quotient may be more than doubled.

After section of the vagi, on the contrary, anoxemia due to carbon monoxide causes no overbreathing, and no distinct lowering of blood alkali, even up to death. This fact, appears to be a decisive demonstration that oxygen deficiency itself does not directly cause in the tissues and blood an increased production of organic acids.

BIBLIOGRAPHY.

1. Araki, T., *Z. physiol. Chem.*, 1891, xv, 335, 546; 1892, xvi, 201, 453; 1894, xix, 422.
2. Ryffel, J. H., *J. Physiol.*, 1909–10, xxxix, p. v, ix, xxix.
3. Saiki, T., and Wakayama, G., *Z. physiol. Chem.*, 1901–02, xxxiv, 96.

4. Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 333, 345, 355, 365.
5. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.
6. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 3, 15. Henderson, Y., 1920, xliii, 29.
7. Haggard, H. W., *Am. J. Physiol.*, 1921 (in press).
8. Haldane, J. S., Kellas, A. M., and Kennaway, E. L., *J. Physiol.*, 1919–20, liii, 181.
9. Macleod, J. J. R., *Am. J. Physiol.*, 1921, lv, 184.
10. Henderson, Y., *Science*, 1919, xlix, 431.
11. Henderson, Y., and Haggard, H. W., *J. Pharmacol. and Exp. Therap.*, 1920, xvi, 11.
12. Henderson, Y., Haggard, H. W., and Coburn, R. C., *J. Am. Med. Assn.*, 1920, lxxiv, 783.
13. Henderson, Y., and Scarbrough, M. M., *Am. J. Physiol.*, 1910, xxvi, 260. Bryant, J., and Henderson, Y., *J. Am. Med. Assn.*, 1915, lxv, 1.
14. Haldane, J. S., Respiration, New Haven, 1921.
15. Henderson, Y., *Am. J. Physiol.*, 1910–11, xxvii, 152; *J. Am. Med. Assn.*, 1916, lxvii, 580.
16. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 77.

ANTIKETOGENESIS.

I. AN IN VITRO ANALOGY *

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For many years the writer has been interested in the "acetone bodies," and various papers dealing with these substances have been published from this laboratory. Our main interest has been in the problems of intermediary metabolism underlying the formation of the acetone bodies; but until about 2 years ago we were not able to secure the sort of evidence which seemed to us essential for the adoption of a conception held in common with certain other workers on the subject, and our progress on these problems was slow. The missing evidence has now been found and with this encouragement the subject of ketogenesis and of the mechanism of antiketogenesis has been taken up and is being developed from several directions.

It has long been common knowledge that if any human subject fasts, or merely omits carbohydrate from his food for a few days, acetone appears in his breath, and acetone and acetoacetic and β-hydroxybutyric acids are excreted in his urine. The same substances are excreted in larger amounts by subjects of severe diabetes and are responsible for the acidosis and coma in this disease.

The precursors of the acetone bodies are known to be chiefly the fats and certain of the amino-acids of protein. And the fact, first pointed out by Hirschfeld (1) in 1895, and since abundantly confirmed, that they appear when the amount of carbohydrate catabolized is small, demonstrates a relationship between carbohydrate metabolism and the production or the avoidance of production, of the acetone bodies. A legion of workers in the past two or three decades has tested a great variety of substances as to their effect in causing or preventing acetone body production and many of these substances have been definitely classed as either "ketogenetic" or "antiketogenetic." But efforts to explain the character of the action of antiketogenetic substances in preventing acetone body formation have not been successful and although there are many hypotheses, there is at present little direct evidence to indicate the mechanism of the reactions which may be involved.

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* A preliminary report of the experiments described in this paper was presented in December, 1919, before the American Society of Biological Chemists; and their application to the determination of the ketogenic antiketogenic balance in man was presented before the Federation of American Societies for Experimental Biology in December, 1920 (*J. Biol. Chem.*, 1921, xlvi, p. vi).

As first suggested by Geelmuyden (2) in 1901, the most likely explanation would appear to be a definite chemical reaction between some one of the acetone bodies, or one of their precursors, and glucose, or a product of its catabolism. The detailed hypotheses of Woodyatt (3) and Ringer (4) as well as of Geelmuyden are based upon this conception, but the reactions proposed by these investigators have not been established and, perhaps in consequence, the underlying conception has not been developed and applied as it deserves. These hypotheses will be again referred to in the following paper.

With the feeling that it should be possible to find an analogy in the test-tube to the antiketogenic action of glucose in the body the writer from time to time has sought for evidence of an effect by glucose upon the oxidation of fatty acids and of the individual acetone bodies by hydrogen peroxide. The results were uniformly negative[1] until the combination of acetoacetic acid and glucose in alkaline solutions was tried. With this combination the results are striking. When hydrogen peroxide is added to such a mixture the acetoacetic acid disappears rather rapidly even at room temperature, the rate of disappearance increasing with the amount of glucose and the alkalinity. In strongly alkaline solution (N NaOH) at body temperature and in the presence of an excess of glucose and hydrogen peroxide the oxidation is complete in a few hours, while under the same conditions except for the absence of glucose, 24 hours or longer are required. In less alkaline solutions the rate of the reaction is slower, though equally striking; while in neutral or acid solution the effect of glucose is absent. With acetone, hydroxybutyric acid, or butyric acid a similar reaction has not been found. Glucose thus exhibits, in alkaline solution *in vitro*, a "ketolytic" action in hastening the oxidation of acetoacetic acid which would appear to be analogous to its "antiketogenic" action in the body. Among other antiketogenic substances so far tried, glycerol and fructose are ketolytic while lactic acid has no such action.

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[1] Experiments of a similar character reported by Witzemann (5) led him to the opinion (6) that the action of glucose in antiketogenesis is to "spare" butyric acid from oxidation and thus *prevent the formation* of acetone bodies. But other evidence is opposed to such a view of the nature of antiketogenesis.

While the degree of alkalinity found to be necessary does not, of course, exist in the body, and one cannot therefore suppose the *in vitro* and *in vivo* reactions to be identical, there are facts suggesting that the alkalinity may concern primarily the "dissociation" or preliminary decomposition of glucose rather than other stages of the reaction, perhaps the same effect to which Woodyatt (7) has directed attention in comparing the action of alkali on sugar with the action of the pancreatic hormone. At any rate, it seems to us that the "ketolytic" action of glucose may profitably be studied on the assumption that it is similar to the reaction taking place in the body. And on the other hand, we are inclined to accept the existence of the *in vitro* reaction as strong evidence in favor of the view that antiketogenesis is to be explained by a chemical reaction between ketogenic and antiketogenic substances. In the following paper we attempt to apply this conception.

In the present paper we shall present experiments which illustrate the ketolytic action of glucose, fructose, and glycerol, and which bring out the effect of alkalinity and temperature upon the reaction. A discussion of the products of the reaction and its chemical mechanism will be postponed to a later paper.

EXPERIMENTAL.

Acetoacetic acid was prepared from the ethyl ester by saponification with 1 or 2 N NaOH at room temperature for 24 to 36 hours, or in the incubator over night. The solution was cooled, acidified with sulfuric acid to Congo red paper, and extracted three to six times with a half volume of ether in a separatory funnel. The ether layer was separated and shaken in a second funnel with a small volume of water containing a few drops of alizarin red and kept alkaline by the addition of NaOH. The keto-acid is quickly and completely absorbed as sodium salt by the water, and the same ether is thus used repeatedly for extraction of the saponified ester solution. The alkaline solution of the salt is aerated by a strong air current for some hours to remove acetone, is filtered, and kept in the ice box. The acid is gradually decomposed into acetone and carbon dioxide, but in alkaline solution at room temperature or below, the rate of decomposition is slow, amounting to less than 1 per cent per day (Engfeldt (8)). When the solutions are boiled, preferably in acid solution, the keto-acid is rapidly and quantitatively decomposed into acetone which may be determined in the distillate by the iodine titration.

In alkaline solution hydrogen peroxide alone very slowly oxidizes acetoacetic acid at ordinary temperatures. At boiling temperature, during distillation the oxidation is more rapid but the rapid decomposition of the acid into acetone, which is quite resistant to peroxide and quickly distills off, prevents great loss. Oxidation during distillation may be wholly avoided by acidifying the mixture before distillation, when neither acetoacetic acid nor acetone is attacked by hydrogen peroxide, and the yield of acetone is quantitative.

The procedure usually followed in the experiments described below was to mix known amounts of sodium or calcium acetoacetate in slightly alkaline solution, sodium hydroxide, hydrogen peroxide, and glucose, some one or more of the components being omitted in various controls. All the mixtures were diluted to the same volume, usually 500 or 1,000 cc., the flasks stoppered and placed at about the same temperature. In the early experiments the solutions were at once heated to boiling and distilled. Under these circumstances the reaction takes place only until, as the temperature rises, the acetoacetic acid is decomposed into acetone and carbon dioxide.

In the later experiments the solutions were allowed to stand at room temperature or in the incubator and from time to time portions were withdrawn and the acetoacetic acid remaining, together with small amounts of acetone formed by the slow ketone decomposition, was determined as acetone after distillation from the acid solution. As stated above the reaction does not take place in acid solution; but since volatile acids and small amounts of hydrogen peroxide may pass into the distillate from acid solutions, the first distillate was usually redistilled from alkaline solution (NaOH) or after the addition of sodium peroxide to remove possible aldehydes. In either case the peroxide is decomposed, and is without action upon acetone.

Experiment 11.—One of the early experiments is recorded in Table I 50 cc. of an approximately 0.4 mol calcium acetoacetate solution were placed in each of four flasks, to which were added solutions of glucose and NaOH as stated in the table, and the mixtures were diluted to 100 cc After 3 days at room temperature, 5 cc portions were diluted to 300 cc and distilled, after adding (*a*) 25 cc. of 5 N H_2SO_4, (*b*) 25 cc. of 3 per cent H_2O_2 and NaOH to make the solution 0.25 N, and (*c*) H_2O_2 and 0.5 N NaOH.

TABLE I.

Effect of Glucose and Alkali on the Oxidation of Acetoacetic Acid by H_2O_2.

Experiment 11. Solutions mixed and distilled after 3 days at 20±°C. Redistilled distillates after adding Na_2O_2.

Solution No.	In 100 cc of solution.			First distillation from						
	Ca aceto-acetate	Glucose	NaOH	H_2SO_4	H_2O_2 ± neutral		H_2O_2 in 0.25 N NaOH.		H_2O_2 in 0.5 N NaOH	
				Acetoacetic acid as acetone						
				Found	Decomposed in distillation					
	millimols	*millimols*	*millimols*	*mg*	*mg*	*per cent*	*mg*	*per cent*	*mg*	*per cent*
I	20	0	0	1,114	16	1.4	138	12.5	118	10.6
II	20	20	0	1,128	60	5.3	460	10.8	694	61.5
III	20	0	20	1,084	72	6.6	220	20.3		
IV	20	20	20	1,064	72	6.7	492	46.2		

The results allow the following conclusions. (1) There was little, if any, disappearance of acetoacetic acid on standing at room temperature with glucose (in absence of peroxide) in either neutral or alkaline solution. (2) On distillation in the presence of hydrogen peroxide in "neutral" solution, 1.4 to 6.7 per cent of the keto-acid was oxidized, while a much larger amount disappeared on boiling with peroxide in alkaline solution. In the absence of glucose, the loss was 10 to 20 per cent, but *in the presence of glucose 40 to 61 per cent of the acetoacetic acid disappeared.* Since the keto-acid quickly decomposes into acetone, which as other experiments show is not similarly destroyed by glucose and peroxide, *30 to 40 per cent of the keto-acid was decomposed through the presence of an equimolecular amount of glucose within the 5 or 10 minutes* taken to warm the solution to boiling and to split off acetone from those molecules of the acid which had not already been converted into a different substance. Other experiments of the same kind but with larger amounts of glucose showed almost complete decomposition of the acid.

Experiment 14. (Table II) Distillation of Ca Acetoacetate, Glucose, and H_2O_2.—50 cc. of acetoacetate solution (equivalent to 0.7 millimol or 41 mg. of acetone) + 25 cc. of 5 N NaOH (= 0.4 N) + 11.5 cc. of 3 per cent H_2O_2 (= 10 millimols) total volume 300 cc. Glucose added as below, heated slowly, and distilled 25 minutes. Distillates redistilled after acidifying with H_2SO_4.

TABLE II

Experiment 14.

Solution No	In 300 cc. of 0.4 N NaOH · 0.7 millimol Ca acetoacetate 10 millimols H_2O_2 and glucose:	Acetone found	Acetoacetic acid oxidized			
			Total	Corrected for effect of H_2O_2 alone.		
					Total.	Glucose
	millimols	*mg.*	*per cent*	*millimols*	*per cent*	*mols*
I	None.	36	12			
II	0.5	18	56	0.31	43	0.6
III	1.0	5	88	0.54	76	0.5
IV	2.5	2	95	0.59	83	0.24
V	5.0	4	90	0.55	78	0.11

The results show that under the relatively unfavorable conditions during heating to boiling and distillation an excess of glucose accomplishes the decomposition of nearly all of the acetoacetic acid, and that 1 millimol of glucose is as effective (for 0.7 millimol of acetoacetate) as a larger amount.

Experiment 18. (*Table III*).—This experiment shows a marked increase in the ketolytic action of glucose with increasing alkalinity of the mixture. 25 cc. of Ca acetoacetate solution (= 0.7 millimol or 41 mg. of acetone) were added to 1 millimol of glucose and 10 millimols of H_2O_2 in a volume of 250 cc., and after adding NaOH to give the final alkalinity stated, the solutions were distilled and the distillates redistilled from dilute H_2SO_4.

TABLE III.

Experiment 18. Immediate distillation.

In 250 cc. of solution: 0.7 millimols Ca acetoacetate 1.0 " glucose. 10.0 " H_2O_2.	Alkalinity NaOH.	Acetone found	Acetoacetate oxidized as acetone	
			Total	
	N	*mg*	*mg.*	*per cent*
I	± Neutral.	41		
II	0.01	36	5	12
III	0.10	28	13	32
IV	0.20	13	28	68
V	0.5	7	34	83

Experiment 34. (*Table IV*) *Ketolytic Action of Glucose at 20°C.*—After mixing as stated in Table IV the solutions stood in stoppered flasks at room temperature (20±°C.) for 16 hours, when they were acidified with H_2SO_4 and distilled. In acid solution both oxidation by peroxide and the effect of glucose are avoided, and the results represent therefore the reaction which took place at room temperature. The distillates were redistilled from Na_2O_2.

TABLE IV.

Experiment 34. 16 hours at 20°C.

Solution No	1.5 millimols Ca acetoacetate in 250 cc 0.2 N NaOH, containing also:	Acetone found	Acetoacetate oxidized
		mg.	*per cent*
I	Control, no H_2O_2, no glucose.	73	
II	22 millimols H_2O_2, no glucose.	67	8
III	22 " H_2O_2 + 2.5 millimols glucose.	19	74

Experiment 44. (*Table V*).—Solutions were mixed as stated below. After standing at room temperature (20±°C.) for 48 hours, 50 cc. of each were acidified, diluted, and distilled. Distillates were redistilled from NaOH to remove volatile acids and peroxide.

TABLE V.

Experiment 44.

Solution No.	In 500 cc. of 0.5 N NaOH: Ca aceto-acetate.	Glucose	H_2O_2	After 48 hours at 20°C.: Acetone found	Acetoacetic oxidized corrected for H_2O_2 alone	For 1 mol glucose
	millimols	*millimols*	*millimols*	*mg*	*mol*	
I	20			1,173		
II	20		100	592	10.0	
III	20	100		958	3.7	
IV	20	4	100	360	4.0	1.0
V	20	10	100	126	6.3	0.6
VI	20	20	100	118	6.4	0.3

TABLE VI.

Experiments 45 and 51. 24 hours at 20°C.

Solution No.	Ca aceto-acetate	Glucose	H_2O_2	Acetone found after 24 hrs	Acetoacetate oxidized: Total.	Corrected for H_2O_2: Total	Per mol glucose
	millimols	*millimols*	*millimols*	*mg.*	*millimols*		
			0.5 N NaOH				
I	6.6	0	0	384	0		
II	6.6	0	100	259	2.15		
III	6.6	1.25	100	204	3.17	1.02	0.8
IV	6.6	2.50	100	167	3.74	1.59	0.6
V	6.6	3.75	100	125	4.46	2.31	0.6
VI	6.6	5.0	100	107	4.77	2.62	0.5
VII	6.6	10.0	100	39	5.95	3.8	0.4
			1.0 N NaOH				
I	6.0	0	35	301			
II	6.0	3.0	35	142		2.74	0.91
III	6.0	5.0	35	117		3.18	0.63

From the results of this experiment it appears that if allowance be made for the amount of acetoacetic acid oxidized by peroxide in the absence of glucose, glucose may accomplish the transformation of *an equimolecular amount of acetoacetic acid* when reacting upon a large excess of the keto-acid (Solution IV). In the mixtures with a larger relative amount of glucose, its apparent effect is less, due in part to the error in assuming the full effect of peroxide alone in these solutions. It may be noted also that a large excess of glucose, even without peroxide (Solution III) caused some disappearance of keto-acid. This has been occasionally noted at high alkalinity, but has not been further investigated.

Experiments 45 and 54. (Table VI).—The procedure was the same as in Experiment 44. The solutions stood 24 hours at room temperature (20±°C).

Experiment 56.—After mixing the solutions as stated in Table VII, they stood at 30±°C. for 20 hours, after which portions of each were distilled from sulfuric acid, and the distillates redistilled from NaOH.

TABLE VII

Experiment 56.

Solution No	Ca aceto-acetate	Glucose	H_2O_2	Acetone found	Acetoacetic oxidized in 20 hrs.		
					Total	Corrected for H_2O_2.	
							Per mol glucose
	millimols	*millimols*	*millimols*	*mg.*	*millimols*	*millimols*	
In 500 cc. of 0.25 N NaOH:							
I	11	0	0	668			
II	11	0	100	412	4.42		
III	11	11	100	78	10.15	5.72	0.52
In 500 cc. of 0.5 N NaOH:							
IV	11	0	0	632			
V	11	0	100	366	4.5		
VI	11	11	100	20	10.5	6.0	0.54

Experiment 59. (Table VIII).—The results of this experiment are plotted in curves in Fig. 1, which shows graphically the relative rate of the decomposition of acetoacetic acid under these conditions, in the presence and absence of glucose.

Experiment 65. (Table IX).—Rate of reaction at 30±°C. The details are given in Table IX and are shown in the form of curves in Figs. 2 and 3. Attention may be called to the following points: (*a*) the slow oxidation of acetoacetic acid in the form of its sodium salt in 0.25 N NaOH by H_2O_2

TABLE VIII.

Experiment 59.

Solution No.	Na acetoacetate + glucose + H_2O_2 at 25± °C						
	In 1,000 cc. of 0.2 N NaOH:			Acetone by distillation.			
	Na aceto-acetate.	Glucose	H_2O_2	Start	3 hrs	6 hrs.	22 hrs.
	millimols	*millimols*	*millimols*				
I	16.8	0	0	968	932	912	956
II	16.8	16.5	0	944	930	908	892
III	16.8	0	100	1,024	942	962	810
IV	16.8	8.2	100	1,068	936	772	386
V	16.8	16.5	100	996	804	588	140
VI	16.8	33	100	1,036	722	352	148

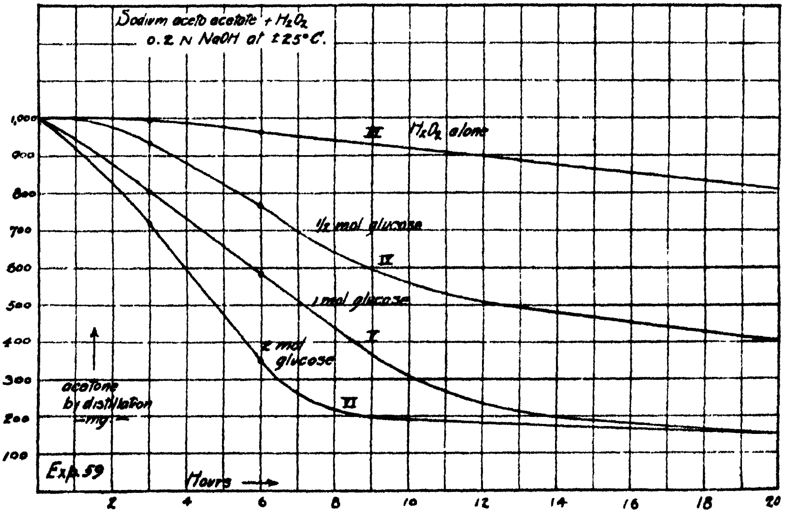

Fig. 1. Experiment 59, showing rate of ketolytic action in 0.2 N NaOH at 25±°C., by 0.5, 1.0, and 2.0 molecular equivalents of glucose.

at 30°C.; (*b*) the increasing speed of its disappearance with increasing amounts of added glucose and with increasing alkalinity (Fig. 2); and (*c*) the fact that an equimolecular amount of glucose (Solution V) was required to accomplish almost complete decomposition, indicating a 1:1 molecular relationship between glucose and acetoacetic acid in the reaction. The slope of the curve with one-half molecular equivalent of glucose is slightly greater than expected and is perhaps due to error in the determination, or the glucose solution added was possibly slightly stronger than was supposed.

Fig. 3 shows the marked increase in the speed of the reaction with increasing alkalinity (Solutions III, V, X, and XI).

TABLE IX.

Experiment 65. Sodium acetoacetate and glucose at 30± °C. At end of period solutions were acidified and distilled. Redistilled from NaOH.

Solution No	In 1,000 cc 0.25 N NaOH:			Acetone recovered by distillation, representing the unoxidized acetoacetate after:						
	Na aceto-acetate	Glucose	H_2O_2	12 min.	25 min	60 min	125 min	235 min	420 min	24 hrs
	milli-mols	*milli-mols*	*milli-mols*							
I	20	0	0		1,204		1,206	1,208		1,148
II	20	20	0		1,204		1,196	1,196		1,148
III	20	0	150		1,212		1,198	1,172	1,120	1,020
IV	20	10	150		1,216		1,164	1,054	812	244?
V	20	20	150		1,198		1,128	916	524	32
VI	20	40	150		1,112		1,004	696	182	38
VII	20	$20D_1$	150		1,118		844	612	352	39
					30 min		150 min		300 min	
VIII	20	$20D_2$	300	1,180	1,112	1,088	1,018		942	
IX	20	$10D_2$	300	1,196	1,154	1,128	1,052		976	
	In 0.5 N NaOH:					80 min		260 min.	375 min	
X	20	20	150	1,202		1,096		488	128	26
	In N NaOH.									
XI	20	20	150	1,204		1,068		138	56	26

Another point of great interest is shown in this experiment. It will be seen that in Mixtures IV, V, and VI (Experiment 65, and Fig. 2) in which neutral, fresh solutions of glucose were added simultaneous with the peroxide, there was an interval of an hour or less before the decomposition or disappearance of acetoacetic acid began, after which, as shown by the shape of the curves, the rate of the reaction increased to a maximum. If, however, the glucose is first allowed to stand for some hours with alkali, during which time various rearrangements and "dissociations" of glucose take place, as shown by the well known work of van Eckenstein, de Bruyn, and Nef (9), the reaction with acetoacetic acid and peroxide begins at once when the last two are added. In Mixtures VII, VIII, and IX of Experiment 65 the glucose used had been previously "dissociated" by standing at room temperature for 16 hours in about 0.3 N NaOH (D_1 in Solution

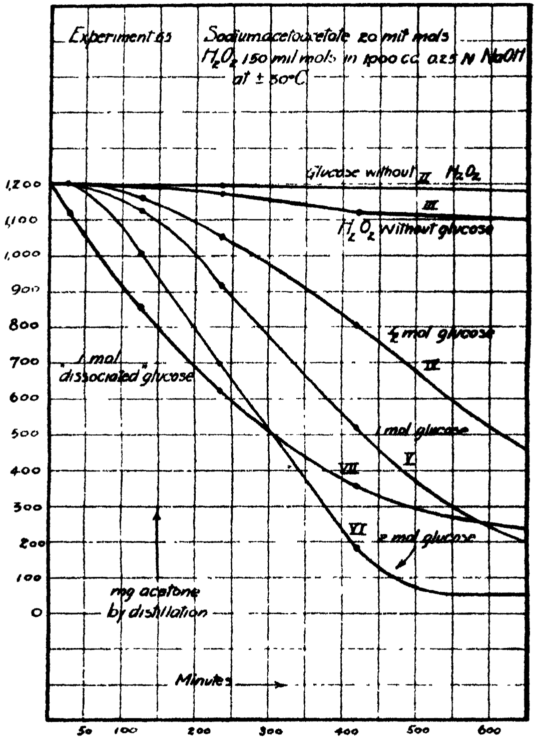

FIG. 2. Experiment 65, showing rate of ketolytic action of ordinary glucose as compared with the faster rate with glucose previously "dissociated" by treatment with alkali.

VII) and 0.6 N NaOH (D_2 in Solutions VIII and IX). For at least 5 hours the rate of reaction of Solution VIII exceeded the rate with double the amount of ordinary glucose, Solution VI, after which time the rate falls off, but continues to exceed that of Solution V containing the same amount of "undissociated" glucose. The same phenomenon is observed in Solutions VIII and IX but in these cases the initial rate more quickly falls off, perhaps due to an injurious effect of the stronger alkali (0.6 N) on the glucose. The results in these experiments are less striking and their curves are omitted from the figure. Similar experiments have been repeated a number of times with essentially the same result. The rate of the oxidation of glucose alone, by peroxide, is likewise affected by previous treatment with alkali, and there appears to be no doubt that the rate of "dissociation" by alkali is one of the limiting factors determining the rate of the reaction with acetoacetic acid.

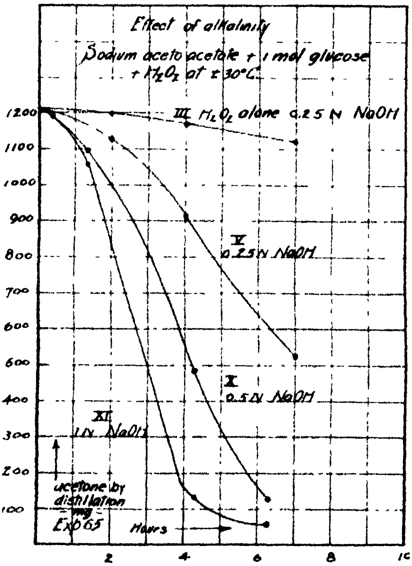

FIG. 3. Experiment 65, showing increasing rate of ketolytic action with increasing alkalinity.

It is evident from this behavior that glucose must be either first "dissociated" or transformed into some other more active substance before it takes part in this reaction. This fact is at least suggestive when it is recalled that glucose is not antiketogenic in the severe diabetic who is unable to metabolize it. One is tempted to wonder whether the diabetic could use glucose after it had been "dissociated" for him by appropriate treatment with alkali. Further consideration of this matter will be reserved for a later paper.

Ketolytic Action of Glycerol, Fructose, and Lactic Acid.

The results given below in Tables X, XI, and XII, and in Figs. 4 and 5, representing Experiments 63, 68, and 69 show that fructose and glycerol have marked ketolytic action, similar to and perhaps even exceeding that of glucose. Both are antiketogenic when fed to diabetics. Lactic acid on the other hand appears not to be capable of affecting the decomposition of acetoacetic acid under the conditions of the *in vitro* experiments. Further experiments with these and other substances are in progress and will be reported in a later paper.

TABLE X.

Experiment 63. *Na Acetoacetate + Fructose + H_2O_2 at 30°C.*

Solution No.	In 250 cc. 0 5 N NaOH:			Acetone by distillation from H_2SO_4 and redistillation from NaOH.		
				Total for 250 cc		
	Na acetoacetate.	Fructose.	H_2O_2	Start	1¾ hrs	3¼ hrs.
	millimols	*millimols*	*millimols*			
I	5	5	100	238	61	39
II	5	0	100	235	226	214
III	0	5	100	11		

TABLE XI.

Experiment 68. *Ketolytic Action of Glycerol and Lactic Acid at 38°C.*

Solution No.	In 1,000 cc. normal NaOH: 20 cc mol Na acetoacetate. 200 " " H_2O_2 and:	Acetone by distillation from H_2SO_4 and redistillation from NaOH.		
		3¼ hrs	5⅜ hrs	23½ hrs
I	Control, H_2O_2 alone.	(1,090)	(1,030)	(550)
II	20 mol glucose.	221	82	93
III	20 " glycerol.	535	148	54
IV	20 " lactic acid.	985	834	442

TABLE XII.

Experiment 69. *Ketolytic Action of Lactic Acid at 38°C.*

Solution No.	In 1,000 cc of normal NaOH: 20 cc. M Na acetoacetate + 200 cc. M H_2O_2, and:	Acetone by distillation from H_2SO_4 and redistillation from NaOH.			
		1 hr.	2 hrs.	3½ hrs.	19 hrs.
	Control, H_2O_2 alone.	1,122		982	547
	20 M lactic acid.	1,148	1,117	1,068	640

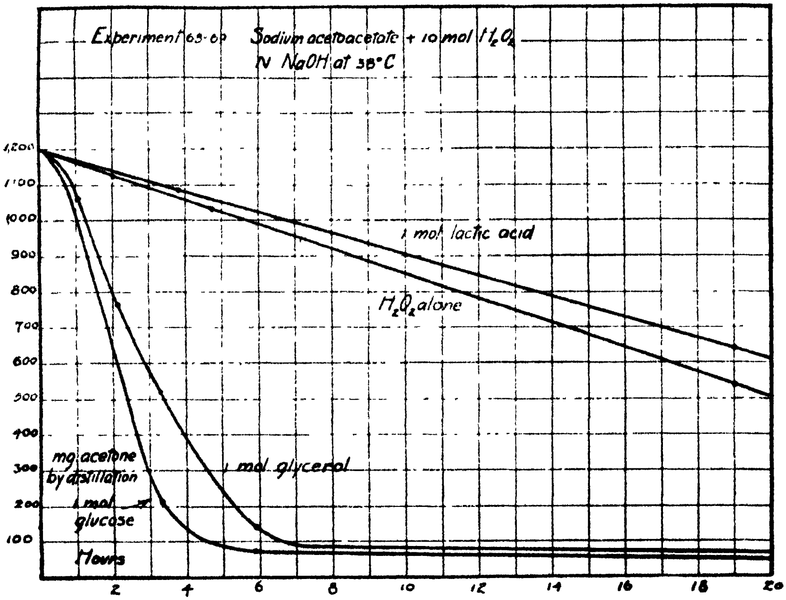

FIG. 4. Experiments 68 and 69, showing rate of ketolytic action of glucose and glycerol, and absence of such action by lactic acid, in normal NaOH at 38±°C.

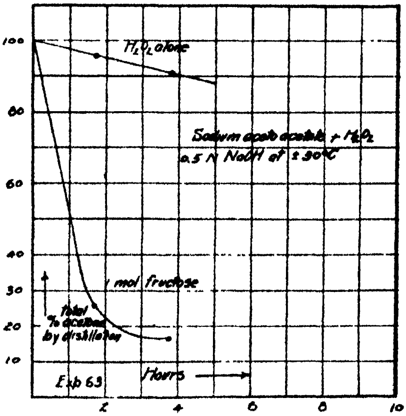

FIG. 5. Experiment 63, showing rapid ketolytic action by fructose in 0.5 N NaOH at 30±°C.

SUMMARY.

The foregoing experiments demonstrate that the oxidation of glucose in alkaline solution by hydrogen peroxide accomplishes the disappearance of acetoacetic acid if the latter be present in the solution. Acetoacetic acid in the absence of glucose or other "ketolytic" substance is oxidized very slowly by hydrogen peroxide, but its disappearance is rapid even at room temperature if glucose is simultaneously oxidized. Fructose and glycerol exert the same effect as glucose, while lactic acid is without such action.

The rate of the "ketolytic action" is increased with alkalinity, temperature, and amount of glucose or other ketolytic substance. The rate of the reaction appears to be determined primarily by the rate of the "dissociation" or the conversion of glucose by alkali into a derivative which is then oxidized. The inference seems justified that it is some intermediate oxidation product of glucose which combines with acetoacetic acid, the compound being then further oxidized. The details of the reaction and its products will be considered in a separate paper.

The phenomenon is believed to be an *in vitro* analogy to the action of glucose and of similar substances in abolishing or preventing the formation (accumulation) of acetoacetic acid and the related acetone and β-hydroxybutyric acid in man.

BIBLIOGRAPHY.

1. Hirschfeld, F., *Z. klin. Med.*, 1895, xxviii, 176.
2. Geelmuyden, H. C., *Z. physiol. Chem.*, 1904, xli, 135.
3. Woodyatt, R. T., *J. Am. Med. Assn.*, 1910, lv, 2109.
4. Ringer, A. I., *J. Biol. Chem.*, 1914, xvii, 107.
5. Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 83.
6. Witzemann, E. J., *J. Am. Chem. Soc.*, 1917, xxxix, 2660.
7. Woodyatt, R. T., *J. Biol. Chem.*, 1915, xx, 129.
8. Engfeldt, N. O., "Beitrage zur Kenntnisse der Bio Chemie der Acetonkorper," Lund, 1920, 80.
9. Nef, J. U., *Ann. Chem.*, 1913, cccciii, 204.

ANTIKETOGENESIS.

II. THE KETOGENIC ANTIKETOGENIC BALANCE IN MAN.

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In the preceding paper (1) it has been shown that glucose and related substances such as fructose and glycerol exert a marked effect upon the oxidation of acetoacetic acid by hydrogen peroxide in alkaline solution. At room temperature or in the incubator, and in the absence of glucose, alkali salts of acetoacetic acid are only very slowly oxidized by hydrogen peroxide; but if glucose is present the disappearance of the keto-acid is rapid, the rate increasing with the temperature, with increasing alkalinity of the solution, and with increasing amounts of glucose. Although the details of the reactions are not yet fully known, it is evident that a chemical reaction occurs between some derivative of glucose and acetoacetic acid, involving definite molecular quantities of each substance. It is likewise evident that this "ketolytic" action of glucose *in vitro* is strikingly similar to the long known "antiketogenic" action of glucose and related substances in preventing the appearance of the acetone bodies in man. Although the degree of alkalinity used in the experiments is not approached in the body, the cells probably possess, as pointed out by Woodyatt (2), some means of producing a similar effect. As explained in the preceding paper, it was the search for a chemical explanation of "antiketogenesis" which brought to light the behavior of glucose there described.

The fact that the ketolytic action of glucose *in vitro* is a chemical reaction suggests that the effect of carbohydrate in preventing or abolishing ketonemia in man is also the result of a definite chemical reaction in the tissues.

The latter conception is by no means new as will be shown by the review of the literature, but the various hypotheses in which such conceptions have been advanced have not been established, and there is at present no accepted explanation of the mechanism of antiketogenesis.

The literature on the subject under discussion is so large that its detailed and complete review will not be attempted, but brief reference to a few of the more important investigations and hypotheses concerning antiketogenic action is necessary.

The relationship between acetonuria and carbohydrate starvation, first pointed out by Hirschfeld (3) and Rosenfeld (4) has been abundantly established. All of the various conditions in which acetone and the related two acids are excreted appear to have in common the fact that abnormally small amounts of carbohydrate are being *burned.* The formation of acetoacetic and β-hydroxybutyric acids does not represent a qualitative disturbance of metabolism; since both are readily oxidized when given to man and animals (5–7), it is more reasonable to believe that they represent normal intermediates in metabolism, which, in the absence of sufficient carbohydrate combustion, escape further oxidation (Geelmuyden, 8). When they accumulate in the tissues, acetone, which is not a normal intermediate, is formed by decomposition of acetoacetic acid (8). Geelmuyden (8) in 1904 first advanced the hypothesis that carbohydrate and acetone bodies react in their intermediary metabolism by a synthesis which is necessary for the further decomposition of the acetone bodies; and that failing this synthesis their accumulation and excretion result. He assumed a conjugation with glycuronic acid as explaining the character of the reaction, and called it "die Hypothese von der chemischen Interferenz der intermediären Stoffwechselproducte." In a later paper, Geelmuyden (8) reiterates this view, but no experimental evidence was adduced as to the qualitative or quantitative aspects of the chemical reactions between the acetone bodies and the derivative of glucose. Rosenfeld (9) proposed the less definite conception that carbohydrate serves to catalize or set off the oxidation of fats, "Der Zundstoff für die Fette sind die Kohlenhydrate."

That such ideas failed to take root is indicated by the remark of von Noorden (10), who, in his Handbuch concludes the discussion of the action of carbohydrate in preventing acetone body formation with the statement, "Wie die Kohlenhydrate und antiketogenen Substanzen es bewirken....wissen wir nicht."

In 1910 Woodyatt (11) clearly formulated the problem and set forth a speculative explanation on the assumption that "antiketogenesis is an effect due to certain products which occur in the oxidation of glucose, an interaction between these products on the one hand and one or more of the acetone bodies on the other." The conception advanced by Woodyatt was suggested by the work of Ciamician and Silber on the reciprocal oxidation and reduction of alcohols and ketones, keto-acids and other substances when exposed to sunlight, and was to the effect that acetoacetic acid, the first formed of the acetone bodies, undergoes a reciprocal reduction by the simultaneous oxidation of glucose, glyceric aldehyde, or other antiketogenic substance. While Woodyatt's assumption above quoted and as later elaborated (12) appears to be supported by the results in the preceding paper, no direct evidence has been advanced concerning the mechanism of the reactions which he proposed.

A third "chemical interference hypothesis" was presented in 1914 by Ringer (13) who conceived "the rôle of glucose in the normal individual in preventing acidosis to be such as to deviate the β-hydroxybutyric acid from its ordinary course of oxidation, by combining with it and thereby changing its structural configuration so as to give rise to non-acetone genetic products." Structural formulas were given of various hypothetical reactions, the experimental basis being the earlier finding by Ringer and Frankel (14) that acetaldehyde and propyl aldehyde when given subcutaneously to diabetic dogs produced a fall in the acetone body excretion and a rise in the sugar excretion. Ringer and Frankel concluded that all aldehydes including glucose react with β-hydroxybutyric acid and that the metabolism of the resulting compounds results not only in the disappearance of β-hydroxybutyric acid but also in the conversion of the latter, in part, into glucose, thus in effect accomplishing the formation of glucose from a derivative of fatty acid.

Later work by Sansum and Woodyatt (15), however, appears to show that the "extra" glucose found in Ringer and Frankel's experiments is due to the sweeping out of sugar from stored glycogen as a result of the narcotic effect of the aldehyde, and thus throws doubt upon Ringer's interpretation. Sansum and Woodyatt conclude that "the hypotheses of antiketogenesis and diabetes which are based on the assumption that acetaldehyde promotes a new formation of sugar from fat are wholly untenable."

In view of the fact that ketonuria in non-diabetic subjects results from a deficient supply of carbohydrate food, and that in the diabetic it is associated with an inability to metabolize carbohydrate, it has long been appreciated that the cause for the formation of the acetone bodies is in both essentially the same. This point of view has been well emphasized by Woodyatt (12); and its acceptance would seem to be necessary if antiketogenesis is of the nature of a chemical reaction between definite molecular quantities of ketogenic and antiketogenic substances. But as a result of experience with the fasting treatment for diabetes advocated by Allen, the view is now expressed that an essential difference does exist between the ketonuria of diabetic and non-diabetic subjects. Joslin (16), for instance, writes:

> "The anomaly becomes greater when it is considered that a healthy individual, fasting or on a non-protein [(?) non-carbohydrate] diet, develops an acidosis, whereas a severe diabetic treated exactly in the same manner loses an acidosis. There is surely something here which is not understood. It would appear that the diabetic individual is able to manufacture and utilize some substance which in its combustion is able to prevent acidosis, but yet a material which is not available to the normal individual."

Furthermore, the view is held that not only do different subjects show different reactions to fasting or carbohydrate starvation, but that an adaptation from training may render a subject more or less immune to the deprivation of carbohydrate. Thus Joslin, under the heading "adaptation of the body to a non-carbohydrate diet and to acidosis" states:

> "It is reasonable to conclude that the body of the fasting man adapted itself to the changed conditions and in some way restricted the output of acid bodies. This may be the explanation of the lack of acidosis among the Eskimos. This power of adaption is certainly an important factor.

Folin and Denis (17) similarly interpret their observations on the ketonuria of two obese women during successive fasts. They state that their results "suggest with regard to the complete oxidation of body fat in starvation that the human organism is capable of at least a certain amount of adaptation and that it is this individual factor rather than the tendency to obesity or the extent of the fat deposits in the body which chiefly determines the onset and the degree of acidosis." And they "therefore concluded that one of the effects of repeated fastings is habituation to the complete oxidation of mobilized body fat, and a consequent retardation of the development of acidosis."

As representing still a different view the statement of von Fürth may be cited. He (18) writes:

> ". . . the distinct 'antiketogenic' influence of carbohydrates . . . . may be fully explained on the basis of an inhibition of fat decomposition because of the combustible material thus introduced into the body. . . . . this makes all other complicated theories entirely superfluous, especially such theories as that of Geelmuyden. . . . ."

If von Fürth means that carbohydrate acts merely by sparing from oxidation an isodynamic amount of fat, his view is incompatible with the fact that the effect of carbohydrate in preventing ketonuria is much greater than its relative caloric value.

It will be evident from the above review that antiketogenesis still awaits a satisfactory explanation. In the hope of being able to obtain new evidence, and stimulated by the results of the *in vitro* experiments described in the preceding paper, the writer has undertaken to analyze certain data from the literature and to conduct experiments of his own for the purpose of answering the question: *Is there a definite and constant mixture of foodstuffs, corresponding to definite molecular proportions of the precursors of the "acetone bodies" on the one hand, and of glucose or related substances on the other, which just suffices to avoid the appearance of the "acetone bodies" in different subjects?* If the existence of such molecular proportions could be established, it would be a convincing argument in favor of the essential similarity of the ketolytic action *in vitro* to the reaction in the body and would doubtless make possible the study and explanation of many factors in the intermediary metabolism of fats and carbohydrates which are now obscure. For if antiketogenesis is the result of a definite chemical reaction between *definite* and *constant* proportions of the reacting substances we should expect different human subjects, whether the normal, the Eskimo, or the diabetic, to differ as to the formation of acetone bodies only as far as the relative amounts of ketogenic and antiketogenic substances metabolized are different.

The writer believes that acetone body production by all human subjects can be harmonized with this point of view, which is essentially the view proposed by Woodyatt (12) in 1916, but not so far established.

Two methods appear to be suitable for the investigation of the question above stated. From the data of respiration experiments it is customary to calculate, by means of the non-protein respiratory quotient, the amounts of fat and carbohydrate as well as of protein being catabolized. By a simple modification of the calculation one may learn the molecular proportions of the three foodstuffs; and from these data one may attempt to calculate the molecular equivalents of precursors of acetone bodies and of the substances which prevent their appearance. In this way it may be possible to learn whether all subjects who are excreting traces of the "acetone bodies" and are, therefore, on the border-line of ketogenesis and of ketonemia, are catabolizing in their bodies as a whole the same molecular proportion of ketogenic and antiketogenic substances, which proportion, if constant, will evidently be near the minimum ratio required to avoid the appearance of "acetone bodies." Methods of calculating such information and the results of their application to the problem will be presented in later papers of this series.

A second method of obtaining the same information is to feed to various subjects diets in which the proportion of protein, fat, and carbohydrate is varied and to calculate the molecular proportions of mixtures which cause the production and excretion of border-line traces of the "acetone bodies."

It is proposed in the present paper to analyze certain data of this kind, taken chiefly from the published work of others. Other similar data of our own experiments will be presented at a later time. Investigations by the same general method are being conducted also in other laboratories as evidenced by the recent papers of Palmer (19) and Woodyatt (20).

If the mechanism of antiketogenesis in the body may be looked upon as similar to the action of glucose in accomplishing the further decomposition of acetoacetic acid *in vitro*, one may suppose that acetoacetic acid is constantly formed in the body from the fatty acids and other ketogenic substances and that if the derivative of glucose and other antiketogenic substances is locally present it reacts with the keto-acid, and the product is further oxidized.

Whenever the rate of formation of acetoacetic acid exceeds the rate of formation of the antiketogenic substance, the excess of the former fails to take its usual course and as it accumulates is in part decomposed into acetone and reduced to β-hydroxybutyric acid, all of which substances are excreted. Acetone appears to be oxidized slowly if at all in the body, whether acetoacetic and hydroxybutyric acids are oxidized without the intervention of antiketogenic (ketolytic) substance cannot be decided at present.

The problem then is to calculate the total molecular amounts of ketogenic and of antiketogenic substances in those diets, the metabolism of which just causes the definite appearance of small amounts of the acetone bodies.

Although existing information scarcely permits an exact calculation, we may make certain assumptions and learn from their application how far the calculations based upon them are in accord with the facts. The assumptions which appear to be justifiable for a first trial, are indicated below. They are of course subject to modification as further facts are established.

Ketogenic.—The main source of acetoacetic acid is the fatty acids, which are assumed to form an equimolecular amount of the keto-acid. Based on this assumption Magnus-Levy (21) calculated a possible production of 36 gm. of hydroxybutyric acid from 100 gm. of fat. Taking 874 as the molecular weight of the mixed body fat one may calculate that 1 gm. of such mixed fat can give rise to $\frac{1}{874} \times 3 = 0.00343$ gm. molecule of ketogenic fatty acid $\times 102 = 0.35$ gm. of acetoacetic acid.

Another source of ketogenic substances is protein, since leucine, phenylalanine, and tyrosine have been found to be convertable into the acetone bodies when fed to diabetics (22), and when added to blood in liver perfusion experiments (23). Assuming that these observations indicate the normal path of catabolism for these amino-acids, the ketogenic value of protein may be calculated from the analysis of ox muscle by Osborne after the method used by Lusk (24) in calculating the theoretical derivation of glucose. The data are given in Table I and show that each gm. of nitrogen is equivalent to approximately 0.010 gm. molecule of ketogenic substance. In the same table are given also the equivalent amounts of the four amino-acids, valine, lysine, histidine, and tryptophane, which are apparently neutral as to ketogenesis.

Antiketogenic.—Carbohydrates are, par excellence, antiketogenic. Until the active derivative of glucose is known we may assume that one molecule of monosaccharide is equivalent to one molecule of the active derivative, and on this basis calculate the antiketogenic value of carbohydrate in terms of molecules of glucose. Each gm. of glucose would thus be equivalent to $\frac{1}{180}$ = 0.00556 gm. molecule; and each gm. of starch to $\frac{1}{162}$ = 0.00618 gm. molecule.

TABLE I.

Amino-Acids in 100 Gm. of Muscle Protein (Osborne's Analysis as Modified by Lusk).*

		Molecular weight.		Remarks.
	gm.		*gm. molecule*	
Leucine	14.3	131	0.109	Form acetoacetic acid.
Phenylalanine	4.5	165	0.027	
Tyrosine	4.4	181	0.024	
Sum for 16.18 gm. of nitrogen			0.160	Ketogenic.
For each gm. of nitrogen			0.010	
Valine	2.0	117	0.017	
Lysine	7.6	146	0.052	Probably neutral as to ketogenesis.
Histidine	4.5	155	0.029	
Tryptophane	(2.0)?	204	0.01	
Sum for 16.18 gm. of nitrogen			0.099	

* Lusk, G., Science of nutrition, Philadelphia, 3rd edition, 1917, 77.

Protein is known to be converted into glucose by the diabetic organism to the extent of approximately 3.6 gm. for each gm.

of nitrogen, and there is no reason to suppose that the sugar-forming amino-acids do not pass through the same stage, glucose *or its derivative*, in the normal individual as well. 1 gm. of urine nitrogen would then correspond to $\frac{3.6}{180} = 0.020$ gm. molecule of glucose.

A third probable source of antiketogenic substance is the glycerol of fat, though the evidence is perhaps not conclusive. When given as such, glycerol is converted into glucose by the diabetic (25), and is antiketogenic (3, 26, 29), but its fate when ingested in the form of glyceride has not been determined. That an increase in the fat metabolized by diabetics appears not to cause an increased excretion of glucose, may indicate that as glyceride it behaves differently. But in view of the definite conversion to glucose when fed as such, its antiketogenic action, and its chemical relationship it seems preferable for the present to include the glycerol of fat among the antiketogenic substances by supposing that in its catabolism it passes through the stage of glucose or its derivative, in which form it is available for reaction with acetoacetic acid.

Its antiketogenic value, calculated in terms of glucose, is as follows.

1 gm. of fat $= \frac{1}{874} = 0.00114$ gm. molecule fat $\div 2 = 0.00057$ gm. molecule of glucose from glycerol.

The above calculations are summarized below.

Ketogenic substance expressed as gm. molecule of precursors of acetoacetic acid.

(*a*) 1 gm. of fat $= \frac{3 \times 1}{874} = 0.00343$ mol.

(*b*) 1 gm. of urine nitrogen = 0.010 mol.

Antiketogenic substance expressed as gm.-molecular equivalents of glucose.

(*c*) 1 gm. of urine nitrogen $= \frac{3.6}{180} = 0.020$ mol.

(*d*) 1 gm. of glucose from carbohydrate $= \frac{1}{180} = 0.00556$ mol.

(*e*) 1 gm. of fat $= \frac{1}{874} \div 2 = 0.00057$ mol.

The sum of the values of (*a*) and (*b*) divided by the sum of the values of (*c*), (*d*), and (*e*) gives the ratio of ketogenic to antiketogenic substance for the mixture metabolized, and if the general conception is correct, this ratio should determine whether or not the subject will form and excrete acetone bodies.

Before applying this calculation to observed data it may be well to state that it is of course the mixture *metabolized* rather than the mixture of foodstuffs eaten which must be considered. It is therefore necessary to determine or reasonably assume the material being *burned*. Also, it is realized that the mixture metabolized is perhaps different in different cells or different parts of the body at any one time, and that the mixture burned in the body as a whole is constantly changing. But the blood carries the same mixture of foodstuffs to all parts of the body and although the mixture varies from time to time the single source of supply probably tends to maintain an approximately uniform mixture of fuel for all cells at a given time.

It must also be pointed out that the relative values given in some of the above calculations are admittedly doubtful. The results to be presented seem to indicate (see p. 469) that the ketogenic factors of fat and of protein are correctly estimated, but the values assigned to the antiketogenic factors will probably need revision. It is quite possible for instance that the two carbon residues from glycocoll and the three carbon residues from other sugar-forming amino-acids may have direct and immediate antiketogenic (ketolytic) action without condensation to glucose, and the same may be true of glycerol. While these and other uncertainties are fundamental and must be decided, they should, I believe, not deter us from the better understanding of the nature of antiketogenesis and the consequent aid in dietetics which the application of such a conception appears to make possible.

Data of Zeller (27).—In a study of the effect of low carbohydrate diets upon protein metabolism, Zeller records the observation that acetone did not appear until the carbohydrate was reduced to 10 per cent, and the fat increased to 90 per cent of the total calories of food supplied (3,200 calories). This 'diet contained 310 gm. of fat and 80 gm. of carbohydrate, and Zeller concluded that one part of carbohydrate is required for complete combustion of four parts of fat.

Lusk (28) calculates from Zeller's data that perhaps each molecule of hydroxybutyric acid requires the presence of a triose molecule; and Woodyatt (12), citing the same data, concludes that "when the mixture of metabolites oxidizing in the body, contains more than three molecules of higher fatty acids to one of glucose, then the body 'smokes' with acidosis compounds like an automobile smokes with too much oil in the cylinders."

I have recalculated Zeller's data for the diet which first produced acetonuria, as follows. The subject's total metabolism may be approximately calculated from his body weight, 80 kilos, height 175 cm. = 1.95 sq. m. (Du Bois).

	calories
1.95 × 40 × 24 hours = basal metabolism	1,872
10 per cent for effect of food . .	187
10 per cent for 8 hour's rest. . .	62
50 per cent for 8 hour's work in the laboratory	310
Estimated total energy exchange	2,431

In view of the previous low carbohydrate diet it is assumed that all of the ingested carbohydrate was burned and that the fat burned is represented by the difference between the total calories and the sum of the calories from protein and carbohydrate.

		calories
Protein (3.67 gm. × 26.5 calories)	=	97
Carbohydrate (80 × 4.1)	=	328
Total		425

2,431 − 425 = 2,006 calories from fat ÷ 9.3 = 216 gm. of fat burned. The ketogenic and antiketogenic balance may then be calculated.

	Ketogenic	Antiketogenic.	Ratio
80 gm. of carbohydrate.		0.445	
Protein, 3.67 gm. of N.	0.0367	0.0634	
216 gm. of fat.	0.741	0.123	
Total.	0.777	0.631	1.23:1

From these results it is evident that this subject first excreted acetone when the molecular ratio of ketogenic to antiketogenic substances burned (as above calculated) somewhat exceeded 1. With a smaller amount of carbohydrate food Zeller states that more acetone was excreted, but he gave no data as to the amount.

Experiments of Lang (29).—Lang reports a series of experiments upon himself, in which he determined the amount of acetone bodies excreted while on different low carbohydrate diets and during fasting. In the discussion he[1] states:

> "When this ratio (of fat to carbohydrate oxidized) rises above a certain definite value, abnormal quantities of acetone bodies appear in the urine. The results . . . . point to the existence of a quantitative relationship, which has a very definite minimum value, between the fat and the carbohydrate utilized to prevent acidosis.
>
> "These substances (acetone bodies) appear in abnormal amounts immediately the ratio of fat to the carbohydrate burnt becomes greater than about 2 to 1." The ratio refers to grams.

I have calculated the molecular ketogenic balance in some of his experiments, of which the following is the best example. He reports the data given below for periods during which the diet was only 60 and 100 gm. of cane-sugar daily.

Day.	Diet.	Total acetone and acetoacetic acid.	Hydroxybutyric acid.	Total nitrogen.
		mg.	*mg.*	*gm*
1	60 gm. sucrose.	21	186	8.5
2	60 " "	45	224	8.0
3	60 " "	79	214	9.7
4	100 " "	6.6		6.9
5	100 " "	9		11.3
6	100 " "	5		9.8

It is evident that the border-line of ketonuria lies between the conditions in the two periods, the first showing small but distinctly abnormal amounts of acetone bodies, whereas with the larger amount of sugar, the acetone excretion is even smaller than observed by Lang when on full mixed diets. The ketogenic antiketogenic balance and the ratio point to the conclusion that the acetone bodies first appear when the molecular ratio, as here calculated, exceeds 1. In the period when ketonuria existed the ratio is 1.25, while it is 0.88 when ketonuria was absent.

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[1] Lang (29), p. 463.

The total energy exchange is estimated by Lang at 2,500 calories. On this assumption we may calculate the materials burned on the last day of each period as follows.

calories
3rd day. 9.7 gm. of urine N × 26.5 = 257
60.0 gm. of sucrose = 258
515
2,500 - 515 = 1,985 calories = 213 gm. of fat burned.

calories
6th day. 9.8 gm. of urine N × 26.5 = 260
100.0 gm. of sucrose = 380
640
2,500 — 640 = 1,860 calories = 200 gm. of fat burned.

	Ketogenic	Antiketogenic	Ratio
	millimols	*millimols*	$\frac{K}{A}$
3rd day, 60 gm. of sugar.			
N, 9.7 gm.	97	194	
Fat, 213 gm	734.	121	
Sucrose, 60 gm. (63 gm. invert sugar)		350	
Total.	831	665	1.25
6th day, 100 gm. of sugar.			
N, 9.8 gm.	98	196	
Fat, 200 gm.	688	114	
Sucrose, 100 gm. (105 gm. invert sugar)		584	
Total	786	894	0.88

Data of Ascoli and Preti (30).—A normal man, weighing at the start about 59 kilos and whose total energy requirement the authors estimated at 33 calories per kilo, was maintained on fat and protein for a period of several months. With changes in the amounts of food protein and carbohydrate the acetone body excretion varied from about 1 to 13 gm. expressed as hydroxybutyric acid.

Toward the end of the experiment increasing amounts of carbohydrate were added and with 45 gm. the excretion of acetone fell to 130 mg. and of hydroxybutyric acid to 100 mg. or less per day. Taking this period as representing the border-line of ketosis, I have calculated the ketogenic antiketogenic balance (day of February 9) as follows.

54 kilos × 33 calories = 1,782 calories for total energy exchange
Urine nitrogen, 12.2 gm. × 26.5 calories = 323 calories from protein
1,459 calories from fat and CH
45 gm. of glucose × 3.76 calories = 169 calories
1,290 calories from fat
1,290 ÷ 9.4 = 139 gm. of fat burned.

	Ketogenic	Antiketogenic	Ratio
	millimols	*millimols*	$\frac{K}{A}$
N, 12.2 gm.	122	244	
Fat, 139 gm.	477	79	
Glucose, 45 gm.		250	
Total	599	573	1.04

For the preceding day, (February 8) with 30 gm. of glucose added to the diet, and when 220 mg. of acetone and about 200 mg. of hydroxybutyric acid were excreted, the balance may be calculated as follows.

calories
Total 1,782
13 gm. of urine, N × 26.5 = 345
1,437
30 gm. of glucose × 3.76 113
From fat = 1,324 ÷ 9.4 = 141 gm. of fat.

	Ketogenic.	Antiketogenic.	Ratio.
	millimols	*millimols*	$\frac{K}{A}$
N, 13 gm.	130	260	
Fat, 141 gm.	483	80	
Glucose, 30 gm.		167	
Total	613	507	1.21

It would appear from these data that the mixture being metabolized when the subject was on the border-line of ketonuria was such as to supply approximately equimolecular amounts of ketogenic and antiketogenic substances.

Dietary of Eskimos.—Reference is occasionally made to the Eskimo as illustrating an acquired ability to oxidize large amounts of fat with little or no carbohydrate. But when one calculates the ketogenic antiketogenic balance of their dietaries the explanation of their reputed tolerance for fat becomes evident. The calculation by Krogh (31) of the data collected by Rink may be cited as representing such dietaries, according to which the average daily diet of an adult Eskimo contains 282 gm. of protein, 135 gm. of fat, and 54 gm. of carbohydrate. The metabolism of such a mixture would not be expected to lead to acetone body production, the ratio being only 0.7. Even without all carbohydrate, the subject could probably remain practically free from acetonuria, the antiketogenic value of the protein at least equalling the ketogenic value of protein and fat, as shown below.

Eskimo Dietary (Krogh).

	Ketogenic	Antiketogenic	Ratio.
	gm molecule	*gm molecule*	$\frac{K}{A}$
Protein, 282 gm. = 45.3 gm. N.	0.453	0.906	
Fat, 135 gm	0.452	0.077	
Carbohydrate, 54 gm.		0.300	
Total	0.905	1.283	0.7

Subject, H. S. K.—We have carried out experiments similar to those cited from the literature, with in general the same results. Only one need be included in this presentation, and this will be stated briefly. The subject was a patient in the surgical service of the Barnes Hospital and was studied in the Metabolism Ward over a period while awaiting operation for a suspected brain tumor. His complaint was gradual loss of vision; no abnormality of metabolism was detected, and as far as the subject of this paper is concerned, the patient may be considered normal. Age 56, body weight 82 kilos. He remained in bed throughout

TABLE II.

Subject H. S. K.

Date.	Diet.			Total metabolism in 24 hrs.	Urine N.	Metabolized			Ketogenic.			Antiketogenic.				Ratio.	Total hydroxybutyric acid excreted.
	Protein.	Fat.	C H			Protein.	Fat.	C H	Protein.	Fat.	Total.	Protein.	Fat.	C H	Total.		
	gm.	*gm.*	*gm.*		*gm*	*gm.*	*gm.*	*gm.*	*millimols*	*millimols*	*millimols*	*millimols*	*millimols*	*millimols*	*millimols*	$\frac{K}{A}$	*gm.*
Dec. 14 (17.5 hrs.)	20	68	27	1,464	8	51	122	27	80	407	487	160	69	150	379	1.28	0.74
" 15	50	179	38	2,048	10.8	69	172	38	108	574	682	215	98	211	524	1.3	0.77
" 16	50	179	63	2,048	(10.0)	64	164	63	100	548	648	200	93	350	643	1.0	0.36
" 17	48	179	75	2,048	8.8	56	162	75	88	542	630	176	92	417	685	0.9	0.18
" 18	Hospital diet.																0.08

the experiment. The resting metabolism was determined daily with the Benedict spirometer unit apparatus (not the portable), by Dr. L. P. Gay, to whom I am indebted for the results.

After about 60 hours fasting, the patient was given for 2 days a diet representing about 10 per cent over his resting metabolism and containing about 10 per cent of the total calories in the form each of carbohydrate and protein, and 80 per cent as fat. It was expected that this diet would cause the continued excretion of small amounts of acetone bodies, which proved to be the case. The urines were collected in short periods and each analyzed for total nitrogen, acetone + acetoacetic acid, and hydroxybutyric acid.

On the 3rd day of the diet at 5.30 p.m., and on the 4th day additional starch was given and the total acetone body excretion dropped immediately from about 20 mg. of acetone per hour to about 4 mg. per hour.

The results are summarized in Table II. In making the calculations of the ketogenic balance the following assumptions are made: (1) That the total energy exchange for the day was 10 per cent higher than the average determined rate of the resting metabolism; (2) that stored glycogen was exhausted and not available; (3) that the carbohydrate fed was burned; and (4) that the protein metabolized is represented by the nitrogen excretion.

The results show that the ketogenic ratio at the time of small but definite acetone body excretion was with this case, like the preceding subjects, in the neighborhood of 1.

If one reviews the data calculated from the experiments of Zeller, Lang, Ascoli and Preti, the Eskimo dietary, and the experiment with subject H. S. K. it is found that metabolism of the mixtures of fat, carbohydrate, and protein in the proportions shown in Table III led to little or no acetone body excretion, and therefore, are examples of metabolic mixtures at the borderline of ketosis and ketonuria.

From these data one is led to draw the conclusions: (1) that the production and excretion of acetone bodies are dependent upon the *relative* amounts of protein, fat, and carbohydrate in the mixture being metabolized at the time; (2) that, as here calculated, the border-line molecular ratio between ketogenic and antiketogenic factors is 1; and (3) that such diets as supply from carbohydrate at least 10 per cent, from protein about 10 per cent, and from fat not more than 80 per cent of the energy required by the subject, will produce little or no ketonuria.

If the argument being developed in this paper is sound, it should be possible to apply the method of calculation not merely to the border-line of ketosis but to results from cases of extreme acidosis and ketonuria as well. I have made a number of such calculations. In most instances they show what is already indicated by a comparison of the $\frac{K}{A}$ ratios and the amounts of total acetone bodies excreted, as given above in Table III; namely, that although the border-line of ketosis falls approximately at a ratio of 1, when the ratio is greater than 1 the excretion expected from the computation often greatly exceeds the amounts actually found. For example, when subject H. S. K. excreted about 0.7 gm. of total hydroxybutyric acid he had a calculated ratio of 1.30, and if the calculations and underlying assumptions were altogether

TABLE III.

Calculated Metabolic Mixture near Threshold of Ketonuria.

Author or subject	Per cent of total calories from:			Ratio	Total hydroxybutyric acid excreted
	Fat.	Protein	Carbohydrate		
				$\frac{K}{A}$	*gm.*
Zeller	82	4	14	1.23	+
Lang	80	10	10	1.25	0.36
	75	10	15	0.88	0
Ascoli and Preti	72	18	10	1.0	±0.3
	74	19	6	1.2	±0.6
Eskimo dietary	48	44	8	0.7	0(?)
H. S. K.	78	14	8	1.28	0.74
	78	14	8	1.3	0.77
	75	13	13	1.0	0.36
	74	11	15	0.9	0.18

correct he should have excreted an amount of hydroxybutyric acid corresponding to the excess of ketogenic over antiketogenic molecules, which on this day amounted to 158 millimols, or 0.158 × 104 = 16.4 gm. total hydroxybutyric acid. The realization thus falls short of the expectation and indicates that some of the assumptions are wrong. The explanation may, of course, be that some ketogenic substance may be oxidized without the intervention of ketolytic substance, but such a conclusion should be accepted only as a last resort, and if it is not possible to interpret the results without such additional assumption.

The necessary corrections and modifications will doubtless require extended investigation and it is not proposed to make such an attempt here; but it may be stated that the corrections required appear to be necessary rather for the relative values assigned to the antiketogenic factors and not for the ketogenic, which latter there is reason to believe are approximately correct.

As evidence for the last statement and in general confirmation of our hypothesis a recalculation is presented of the very interesting case of severest diabetes, "Cyril K," studied and reported in admirable detail by Gephart, Aub, Du Bois, and Lusk (32).

Severest Diabetes. "Cyril K" of Gephart, Aub, Du Bois, and Lusk.

During a few days this subject was a total diabetic and the authors state that "during this period . . . . all the glucose derivable from protein and all the beta-oxybutyric acid formed as an end-product of the oxidation of fat is completely eliminated in the urine." They further state that "a considerable origin of beta-oxybutyric acid from (amino acids of protein) is not indicated." But according to my calculations of their data, the amounts of total hydroxybutyric acid excreted correspond fairly well with the expectations based on the assumptions, stated earlier in this paper, as to the ketogenic value of protein as well as fat.

Due to the fact that the subject during several days metabolized very little of the sugar-forming material from glycerol as well as from protein, he was thus almost wholly deprived of antiketogenic effect, and in consequence virtually all of the ketogenic molecules catabolized appeared as acetone and acetoacetic and hydroxybutyric acids. And since antiketogenic effect is almost absent, errors in its calculation are also nearly excluded and we are enabled to test the accuracy of the computation of ketogenic factors alone. The results are on the whole quite in harmony with our estimates of the relative ketogenic values of fat and protein.

The statement above quoted from Lusk and his coworkers, as to the origin of hydroxybutyric acid from protein, is based upon calculations from the respiratory quotients, which may be not wholly reliable in severe acidosis. For this reason it has seemed preferable in my computations of these data not to depend upon the respiratory quotients. The method of making the calculations may be illustrated by the following example representing the first day (December 15) on which the subject was studied in the calorimeter.

The hourly metabolism is given as 81.9 calories which for 24 hours would be 1,966 calories. The total acetone body excretion was about 80 gm. of hydroxybutyric acid, (70.9 gm. of hydroxybutyric + 5.61 gm. of acetone and diacetic acid) allowing for the excretion of a few gm. of acetone in the breath. The caloric value of this amount, 80 × 4.69 = 375 calories, is added to the heat produced, giving 2,341 calories. The heat produced from the non-carbohydrate quota of protein is, 36.4 gm. of urine nitrogen × 12.97 calories = 472 calories leaving 1,869 calories derived from the combustion of fat and carbohydrate, including glucose from protein. The amount of sugar burned is estimated as follows:

Urine glucose − food CH = glucose excreted from protein and glycerol
167.9 gm. − 24 gm. = 143.9 gm.
Glucose possible from protein = 36.4 × 3.6 = 131 gm.
"Extra glucose," from glycerol = 12.9 gm.

According to this calculation the subject failed to use, and excreted, not only all of the glucose from protein but 12.9 gm. from glycerol. The caloric equivalent of the latter must be added to 1,869 giving 1,917 calories, equivalent to 203 gm. of fat which was metabolized (incompletely). From these data the ketogenic balance is tabulated.

	Ketogenic	Antiketogenic	Remarks.
	millimols	*millimols*	
N, 36.4 gm.	364	0	All glucose excreted.
C H	0	0	No food C H burned.
Fat, 203 gm .	696	45	116—(12.9 × 5.56) for glucose excreted from glycerol.
Total . . .	1,060 – 45 = 1,015 excess ketogenic.		

1.015 × 104 = 105 gm. of total hydroxybutyric acid expected.

About 80 gm. were actually excreted. It will be noted that glucose corresponding to all of the fat catabolized is 21 gm. of which 12.9 gm. or 61 per cent were excreted. This condition continued during the next 2 days after which, with the onset of fasting, there was a sudden, though small, increase in the sugar-burning power which, coinciding with the fall in total metabolism and the amount of fat burned, resulted in the gradual approach toward ketogenic balance, until on the last day only 11 gm. of hydroxybutyric acid were excreted.

The details of the calculations for other days are given in Table IV and from this the figures representing the ketogenic balance are brought together in Table V. From these data it will be seen that the basis used for the calculation of the total ketogenic and antiketogenic factors accounts approximately for the total hy-

TABLE IV

Results from Severe Diabetic, "Cyril K."

Date	Food C H	Urine N	Urine glucose	Possible glucose from protein (N×3 6)	Urine glucose – food C H	Glucose from protein burned	"Extra" glucose excreted (from glycerol).	Total hydroxybutyric acid excreted	
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm.*	*gm.*	*calorie value*
Dec. 15	24	36.4	167.9	131	143.9	0	12.9	80±	375
" 16.	0.4	38.3	153.4	138	153	0	15.0	80±	375
" 17.. ...	0.4	36.3	140.3	130.7	140	0	9.3	87+	408
" 18.. ..	Fast.	20	55.1	72	55	17	0	58.5+	275
" 19	"	16.7	44.3	60	44.3	16	0	56.8+	267
" 20.....	6	14.1	35.3	50.8	29.3	21.5	0	41.2+	193
" 21......	6.6	14.4	39.7	51.8	33.1	18.7	0	26.2+	122
" 22......	11.4	18.3	26.0	65.9	14.6	51.3	0	11.0+	52

TABLE IV—*Concluded.*

Ketogenic Balance of "Cyril K."

Date.	Total calories liberated		Calories from			Fat catabolized.	Ketogenic millimols.			Antiketogenic millimols.		
			Non C H quota of protein.	Protein glucose burned	Fat burned.		Protein	Fat	Total.	Protein.	Fat	Total.
	per hr.	*per 24 hrs*				*gm*						
Dec. 15	81.9	1,966	472	0	1,917	203	364	696	1,060	0	116 − 72 = 44	44
" 16 . . .	76.4	1,833	497	0	1,767	187	383	642	1,025	0	107 − 83 = 24	24
" 17		(1,800)	471	0	1,772	187	363	642	1,005	0	107 − 52 = 55	55
" 18.....	73.2	1,757	260	64	1,708	181	200	620	820	94	103	197
" 19 . . .		(1,670)	216	60	1,661	175	167	600	767	89	89	178
" 20. . . .	66.3	1,590	183	81	1,519	160	141	549	690	120	120	240
" 21.....		(1,550)	187	70	1,415	150	144	515	659	104	104	208
" 22.....	62.8	1,507	237	193	1,129	119	183	408	591	285	68	353

TABLE V.

Summary of Ketogenic Balance of "Cyril K."

Comparison of hydroxybutyric acid excretion with calculated expectation.

Date.	Excess ketogenic.	Expected hydroxybutyric acid.	If protein and glycerol act as triose.		Hydroxybutyric acid actually excreted.
			Excess ketogenic.	Expected hydroxybutyric acid.	
	millimols	*gm*	*millimols*	*gm.*	*gm.*
(1)	(2)	(3)	(4)	(5)	(6)
Dec. 15.............	1,016	105	972	101	80±
" 16.............	1,001	104	977	102	80±
" 17.............	950	99	895	93	87+
" 18.............	623	65	426	44	58+
" 19.............	589	61	411	43	57+
" 20.............	450	47	210	22	41+
" 21.............	451	47	243	25	26+
" 22.............	238	25	0	0	11+
Totals............		559		436	440+

droxybutyric acid formed and excreted. The greatest discrepancy is about 25 gm. and this would become less if the amount of acetone exhaled were included. The figures indicate, in the writer's opinion the following points: (1) that each molecule of fatty acid gives rise to one molecule of acetoacetic acid. If more than one molecule were formed the actual excretion would have greatly exceeded the expectation. (2) That protein is markedly ketogenic, the production from fat (fatty acid) alone being quite inadequate to account for the amounts of acetone bodies formed. (3) No other source of acetone bodies is indicated, and (4) consequently, the method of calculating the ketogenic factors is approximately correct.

But as already suggested, the method of calculating the antiketogenic influences is less satisfactory. In Table V are given the expected excretion (formation) of total acetone bodies, as gm. of hydroxybutyric acid, calculated (in Column 3) on the assumption used in the preceding calculations, that the active antiketogenic (ketolytic) substance from protein and glycerol as well as from carbohydrate is a six carbon derivative of glucose; and in Column 5 the expected excretion is calculated on the assumption that glycerol and the antiketogenic amino-acids are active in the form of three carbon derivatives. In the latter case the antiketogenic value of protein and of fat would be twice the values given on page 457. While the writer prefers to reserve an opinion on this point, it appears that the latter assumption more nearly coincides with the facts. The total amount excreted during the period (exclusive of acetone in breath, and of acetone and acetoacetic acid on 6 of the 8 days) was 440 gm. The total expected on the first assumption is 559 gm. and on the second assumption 436 gm. In view of the fact that the total metabolism and the amount of fat actually catabolized probably were in excess of the resting periods in calorimeter, which were used as the basis of calculation, one would expect the amounts of acetone bodies excreted to be greater and not less than the calculated amounts. From this point of view the second assumption is the more likely. Also it may be recalled that the *in vitro* ketolytic effect of glycerol is probably the same as an equimolecular amount of glucose. The question is being further investigated.

In view of the many opportunities for error, the moderately close agreement by either method is considered rather remarkable, and is at least strong evidence in support of the underlying conception as to the nature of antiketogenesis.

SUMMARY AND CONCLUSIONS.

Starting with the hypothesis that the property possessed by carbohydrate and other substances of preventing the appearance of acetone bodies (the phenomenon of "antiketogenesis"), is due to a chemical reaction in the body between definite and constant amounts of "ketogenic" and "antiketogenic" compounds, analogous to the "ketolytic" reaction between acetoacetic acid and glucose described in the preceding paper, a trial method has been developed for the calculation of the molecular amounts of ketogenic and of antiketogenic substances derivable from protein, fat, and carbohydrate.

The application of this calculation to various subjects who were excreting small amounts of acetone bodies, indicates that the general hypothesis is correct and that the minimum molecular ratio of ketogenic to antiketogenic substances for the avoidance of ketonuria in different human subjects is 1.

This conclusion is confirmed by the calculation of data from a case of "total" diabetes with extreme acidosis, the total hydroxybutyric acid excretion being approximately accounted for and in fair agreement with the calculated expectation.

There appears to be no reason for believing that any factors other than those concerned with this ratio influence acetone body formation. No difference is to be expected in the behavior of different human subjects, whether normal or diabetic, as regards acetone body formation, except as accounted for by the excess of ketogenic over antiketogenic molecules in the mixture being catabolized.

BIBLIOGRAPHY.

1. Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 433.
2. Woodyatt, R. T., *J. Biol. Chem.*, 1915, xx, 129.
3. Hirschfeld, F., *Z. klin. Med.*, 1895, xxviii, 176.
4. Rosenfeld, G., *Centr. inn. Med.*, 1895, xvi, 1233.
5. Schwartz, L , *Arch. exp. Path. u. Pharmakol.*, 1898, xl, 185.

6. Geelmuyden, H. C., *Skand. Arch. Physiol.*, 1901, xi, 114.
7. Waldvogel, R., Die Acetonkörper, Stuttgart, 1903, 236.
8. Geelmuyden, H. C., *Z. physiol. Chem.*, 1904, xli, 135; 1909, lviii, 255.
9. Rosenfeld, G., *Berl. klin. Woch.*, 1906, xliii, 978.
10. von Noorden, C., Handbuch der Pathologie des Stoffwechsels, Berlin, 1st edition, 1906, ii, 73.
11. Woodyatt, R. T., *J. Am. Med. Assn*, 1910, lv, 2109.
12. Woodyatt, R. T., *J. Am. Med. Assn*, 1916, lxvi, 1910.
13. Ringer, A. I., *J. Biol. Chem.*, 1914, xvii, 107.
14. Ringer, A. I., and Frankel, E. M., *J. Biol. Chem.*, 1913-14, xvi, 563.
15. Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 14.
16. Joslin, E. P., The treatment of diabetes mellitus, Philadelphia and New York, 2nd edition, 1917, 162.
17. Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxi, 183.
18. von Fürth, O., The problems of physiological and pathological chemistry of metabolism, translated by Smith, A. J., Philadelphia and London, 1916, 439.
19. Ladd, W. S., and Palmer, W. W., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 109.
20. Woodyatt, R. T., Paper read before the American Association of Physicians, 1921.
21. Magnus-Levy, A., *Arch. exp. Path u. Pharmakol.*, 1901, xlv, 433.
22. Baer, J., and Blum, L., *Arch. exp Path. u. Pharmakol.*, 1906, lv, 89; 1907, lvi, 92.
23. Embden, G., *Beitr. chem. Physiol. u. Path.*, 1908, xi, 348. Embden, G., Salomon, H., and Schmidt, F., *Beitr. chem. Physiol. u. Path.*, 1906, viii, 129.
24. Lusk, G., *Arch. Int. Med.*, 1915, xv, 939.
25. Cremer, M., *Munch. med. Woch.*, 1902, xlix, 944. Lüthje, H., *Deutsch. Arch. klin. Med.*, 1904, lxxx, 98.
26. Satta, G., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 376.
27. Zeller, H., *Arch. Physiol.*, 1914, 213.
28. Lusk, G., Science of nutrition, Philadelphia, 3rd edition, 1917, 271.
29. Lang, R. M., *Biochem. J.*, 1915, ix, 456.
30. Ascoli, G., and Preti, L., *Biochem. Z.*, 1910, xxvi, 55.
31. Krogh, A., and Krogh M., Diet and metabolism of Eskimos, Meddelelser om Gronland Kommissionen for Ledelsen af de Geologiske og Geografiske Undersogelser i Gronland, Copenhagen, 1914.
32. Gephart, F. C., Aub, J. C., Du Bois, E. F., and Lusk, G., *Arch. Int. Med.*, 1917, xix, 908.

A SIMPLE TECHNIQUE FOR THE DETERMINATION OF CALCIUM AND MAGNESIUM IN SMALL AMOUNTS OF SERUM.

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Method.

1 or 2 cc. of serum are measured into an ordinary 15 cc. graduated centrifuge tube containing 2 cc. of water. 1 cc. of a saturated solution of ammonium oxalate is then added and the sample mixed. The mixture is allowed to stand for half an hour. The volume is then made up to 6 cc. with distilled water and the sample again mixed. The tube is centrifuged at 1,300 revolutions per minute for 10 minutes. All but 0.3 cc. of the supernatant fluid is syphoned off. The precipitate is mixed with the remaining fluid and 2 per cent ammonia (2 cc. of concentrated ammonia to 98 cc. of water) is added up to 4 cc. and mixed. The tube is then centrifuged for 5 minutes. All but 0.3 cc. of the supernatant fluid is again syphoned off. The last step is repeated twice, making three washings in all. The tube is centrifuged for 5 minutes after each washing. After the last washing the supernatant fluid is syphoned off. The crystals are suspended in the residual liquid and dissolved in 2 cc. of approximately N sulfuric acid, heated in the water bath for a few minutes, and then titrated to a definite pink color, which persists for at least 1 minute, with 0.01 N potassium permanganate. This is delivered from a micro-burette graduated in 0.02 cc.

Calculations.

The number of cc. of 0.01 N potassium permanganate used − the blank (volume of 0.01 N potassium permanganate required to produce the same intensity of color in an equal volume of water) × 0.2 = the number of mg. of calcium in the sample.

Reagents.

The only reagent that must be quantitatively accurate is the 0.01 N sodium oxalate (Sörensen). This is used to standardize the permanganate. An 0.1 N sodium oxalate is prepared in the usual way. Solution of the oxalate is facilitated by the addition of 5 cc. of concentrated sulfuric acid. The solution is then diluted 10 times to make an 0.01 N sodium oxalate. The latter solution will remain unchanged for several months; the former indefinitely. The other reagents are prepared in the usual way.

The Magnesium Determination.

5 cc. of the supernatant fluid from the calcium determination corresponding to 1.66 cc. of serum are measured into a 30 cc. beaker, 1 cc. of $(NH_4)_2HPO_4$ solution is added and then 2 cc. of concentrated ammonia. The next day the sample is filtered through a well packed Gooch crucible, washed ten times with 5 cc. of 10 parts of concentrated ammonia to 90 parts of water, then twice with 95 per cent alcohol made alkaline with ammonia. The crucible is returned to the beaker and dried for a few minutes at 80° C. in the oven.

10 cc. of 0.01 N HCl are added to the crucible and after a few hours the entire material is transferred to a test-tube, centrifuged, and 5 cc. of the supernatant fluid are measured into a flat bottomed colorimeter tube graduated for 10 cc., which contains 2 cc. of the iron thiocyanate solution. The volume is then made up to 10 cc. with 0.01 N HCl, a rubber stopper inserted, and the fluid mixed. A series of standards is prepared by adding varying amounts of a known NH_4MgPO_4 solution to the thiocyanate solution and bringing the volume up to 10 cc. as in the unknown samples. The color is compared by looking through the entire length of the liquid column against a white background.

Calculation.—The calculation is the same as in the original method: Reading (cc. of standard solution) $\times$ 0.01 $\times$ 2 $\times \frac{6}{5} \times$ 50 = mg. of magnesium in 100 cc. of serum when 2 cc. of serum are used.

Preparation of Reagents.

Ammonium Magnesium Phosphate Standard.—This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium phosphate ($MgNH_4PO_4.6H_2O$) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. Of this solution 1 cc. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions.[1]

Ammonium Phosphate Solution.—This solution is made as follows: 25 gm. of $(NH_4)_2HPO_4$ are dissolved in 250 cc. of H_2O. 25 cc. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc. This solution is diluted 5 times with water.

The Ferric Thiocyanate Solution.—The ferric thiocyanate solution is made from two solutions which are mixed an hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. 5 cc. portions of Solutions A and B are mixed and the whole is diluted to 40 cc. with water.

10 Per Cent Ammonia.—100 cc. of concentrated ammonia are diluted to 1 liter.

Protocols.

Solutions of blood salts containing calcium and magnesium in concentrations comparable to those found in serum and as high as 30 mg. of phosphorus per 100 cc. of solution in the form of *o*-phosphoric acid have been analyzed by this method and the calcium recovered within 5 per cent of the amount actually present (Table I).

Table II gives the results of a series of comparative determinations on normal and pathological sera performed by this method and by another method previously described by us, the accuracy of which has been established (1).

Table III gives the results of the estimation of magnesium in the supernatant fluid derived from the determination by either calcium method. The figures obtained show excellent agreement.

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[1] Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.

DISCUSSION.

The use of ammonium oxalate for the precipitation of calcium is a well known procedure. Its application for the direct determination of calcium in unashed serum was used by Pribram (2) as early as 1871 and more recently by both de Waard (3) and Clark (4). The former has reported only a few determinations on cow's serum. These are 10 to 15 per cent lower than the results obtained by Meigs, Blatherwick, and Cary (5), Halverson and Bergeim (6), and Marriott and Howland (7) on material derived from similar sources. Thus de Waard reports the finding of

TABLE I.

Analyses of Samples of Solution B.

Specimen No	Calcium.	
	Found.	Present.
	mg	*mg*
1	0.102	
2	0.098	
3	0.096	
Average	0.098	0 100
4	0.198	
5	0.206	
6	0 200	
Average . . .	0.202	0.200
7	0 294	
8	0.290	
9	0.302	
Average...........	0.296	0.300

Composition of Solution B.

NaCl	7.739	gm.
$Na_2HPO_4 + 2\ H_2O$	2.005	“
KCl	0.453	“
$CaCO_3$	0.250	“
$MgSO_4 + 7\ H_2O$	0.189	“
Concentrated HCl	10.0	cc.
Water to	1,000.0	“

8½ mg. of calcium per 100 cc. of cow's serum whereas all the investigators above mentioned have found approximately 10 mg. in the same volume of serum.

Clark (4) has briefly described methods for the direct determination of calcium in plasma and whole blood, using ammonium oxalate which has been titrated to a pH of 7.4 as the precipitating reagent. No figures are published to support the claims made for these methods. In a subsequent communication dealing with the effect of the administration of calcium salts to rabbits, results are reported for the calcium content of plasma and whole blood but the methods used are not described.

TABLE II.

Comparative Calcium Determinations on Serum by the Simplified Technique and the Authors' Original Method.

Specimen No	Diagnosis	Ca per 100 cc. of serum	
		Simplified method.	Authors' original method
		mg	*mg*
1	Normal adult.	10.0	9.6
2	" "	10.5	10.6
3	" "	9.6	9.2
4	" "	9.6	9.5
5	" "	9.9	9.9
6	" "	9.5	9.7
7	" "	10.3	10.1
8	" "	10.3	10.3
9	" "	9.8	10.1
10	" "	10.0	10.0
11	" 11 years old.	9.7	9.7
12	" 9 " "	10.5	11.1
13	Scurvy.	10.2	9.7
14	Diabetes mellitus.	10.7	10.5
15	Pyelonephritis.	10.9	10.3
16	Rickets	9.8	9.5
17	"	8.0	7.7
18	"	7.8	7.4
19	Tetany.	7.5	7.5
20	"	7.3	7.7
21	"	6.2	6.3
22	"	5.7	5.5
23	Chronic nephritis.	7.3	7.8

In a subsequent paper we shall discuss in detail the sources of error in the different methods that have been described for the determination of calcium in serum, plasma, and blood. In the present communication we merely desire to place on record a simplified technique for the quantitative determination of this element in small amounts of serum which has been in use in this laboratory for almost a year and has given satisfactory results when tested on a large series of both normal and pathological sera. Some of these results are published in Table II.

TABLE III

Determination of Magnesium in Serum.

Name	Age	Diagnosis	Mg per 100 cc of serum.	
			Simplified method.	Authors' original method
			mg	*mg*
E. B.	1 yr.	Normal.	1.8	1.8
L. B.	1 "	Tetany (treated).	1.8	1.8
L. B.	1 "	" "	2.4	2.1
B. K.	Adult.	Normal.	2.1	2.2
S. G. R.	"	"	2.2	2.1
F. F. T.	"	"	2.1	2.5
B. K.	"	"	2.1	1.9
F. F. T.	"	"	2.1	2.2
B. B.	"	"	2.3	2.3
G. M.	2 yrs.	Rickets.	2.2	2.2
R. G.	18 mos.	Epilepsy.	1.8	1.8
C. L. D.	14 "	Scurvy.	3.0	2.8

CONCLUSIONS.

A simple and rapid technique for the quantitative determination of calcium in 1 or 2 cc. of serum has been described. The supernatant fluid from this determination may be used for the quantitative determination of magnesium. These methods are accurate to within ± 5 per cent of the amount of calcium and magnesium present.[2]

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[2] Dr. von Meysenbug (personal communication) has compared this calcium method with that of Lyman and obtained excellent agreement.

BIBLIOGRAPHY.

1. Kramer, B., and Tisdall, F. F , *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 44.
2. Pribram, R., Berichte über die Verhandlungen der Königlich Sächsischen Gesellschaft der Wissenschaften zu Leipzig, *Math.-phys. Classe*, 1871, xxiii, 279.
3. de Waard, D. J., *Biochem. Z.*, 1919, xcvii, 186.
4. Clark, G. W., *Proc. Soc. Exp. Biol. and Med* , 1919-20, xvii, 136; *J. Biol. Chem.*, 1920, xliii, 89
5. Meigs, E. B., Blatherwick, N. R , and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.
6. Halverson, J O , and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159.
7. Marriott, W. McK., and Howland, J , *J. Biol Chem.*, 1917, xxxii, 233.

VITAMINE STUDIES.

VIII. THE EFFECT OF HEAT AND OXIDATION UPON THE ANTISCORBUTIC VITAMINE.*

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(Received for publication, June 7, 1921.)

During the summer of 1918 experiments were conducted in this laboratory which indicated that rhubarb juice and orange juice could be boiled for 15 minutes without appearing to lose their antiscorbutic potency. At the time, we were inclined to the belief that the natural acidity of these juices acted in a protective manner. Delf,[1] working with juices extracted from fresh cabbages, Swedish turnips, and oranges, made similar observations She suggested that the stability of the antiscorbutic vitamine, to temperatures above 100°C., might be due to the absence of air.

Other workers [2, 3, 4, 5,] have studied the influence of acidity and alkalinity upon the stability of the antiscorbutic vitamine and are in general agreement that the vitamine is stable in acid solution, while alkalinity tends to destroy it.

Rossi[6] has shown that guinea pigs develop scurvy and die in about 20 days when subsisting upon a diet of hay and oats which

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[1] Delf, E. M., *Biochem. J.*, 1920, xiv, 211.

[2] Weill, E., and Mouriquand, G., *J. physiol. et path. gén.*, 1918, xvii, 849.

[3] Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1919, xxxviii, 293.

[4] Harden, A., and Zilva, S. S., *Lancet*, 1918, ii, 320.

[5] Lamer, V. K., Campbell, H. L., and Sherman, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 122.

[6] Rossi, G., *Arch. fisiol.*, 1918, xvi, 125, cited in *Chem. Abstr.*, 1920, xiv, 1844.

has been subjected to open sterilization. When the same materials are sterilized in hermetically sealed jars guinea pigs grow well and remain healthy for 60 days or more.

Recently, Hess and Unger[7] and Zilva[8] have shown in a more conclusive manner that oxidation has a decidedly destructive action upon solutions containing the antiscorbutic vitamine. The last mentioned writer also observed that carbon dioxide did not inactivate lemon juice, while air possessed a very destructive action. This agrees with our findings with regard to the influence or carbon dioxide and air upon the antiscorbutic potency of heated milks.[9]

Ellis, Steenbock, and Hart[10] have shown that the antiscorbutic vitamine is susceptible to oxidizing agents although they were unable to prevent its destruction in cabbage when this foodstuff was desiccated for 35 hours at 65°C. in an atmosphere of carbon dioxide. The last mentioned observation is difficult to explain unless the destructive action was brought about by intracellular oxidation during the drying process.

EXPERIMENTAL.

The experimental technique in this experiment was similar to that described in previous papers published from this laboratory, with the exception that the scorbutic diet consisted of unhulled oats (60 per cent) and chopped alfalfa hay (40 per cent). The alfalfa hay was autoclaved for 30 minutes at 15 pounds pressure and dried before mixing with the oats. 3 cc. of orange juice, treated in various ways, as indicated in Table I, were fed (daily) to each animal. Hydrogen peroxide was used as the oxidizing agent.

In all cases, where hydrogen peroxide was used, 10 cc. of hydrogen peroxide (3½ per cent) were added for each 100 cc. of fresh orange juice. Those samples of orange juice which did not receive treatment with hydrogen peroxide received 10 cc. of distilled water in order to make all samples comparable as to volume.

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[7] Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 143.

[8] Zilva, S. S., *Lancet*, 1921, i, 478.

[9] Anderson, E. V., Dutcher, R. A., Eckles, C. H., and Wilbur, J. W., *Science*, 1920–21, liii, 446.

[10] Ellis, N. R., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1921, xlvi, 367.

The pasteurized samples (63°C.) were heated in closed flasks in a water bath, while the boiled samples were boiled under reflux condensers to prevent loss by evaporation. All samples were prepared at the same time and under the same conditions. Sufficient quantities were prepared at one time to last for 1 week. After treatment all samples were cooled in running water and placed in a refrigerator. The containers were made of Pyrex glass, tightly stoppered to exclude the air.

TABLE I.

Experimental Diets.

Experimental group	Diet
I	Basal diet + 3 cc. of raw orange juice
II	" " + 3 " " orange juice + H_2O_2 (room temperature).
III	" " + 3 " " " " heated for 30 minutes (63°C.).
IV	" " + 3 " " " " + H_2O_2 heated for 30 minutes (63°C.).
V	Basal diet + 3 cc. of orange juice boiled for 30 minutes
VI	" " + 3 " " " " + H_2O_2 boiled for 30 minutes.

DISCUSSION.

The results of the experiments are indicated in Charts I, II, and III. All the animals receiving 3 cc. of raw orange juice (Group I, Chart I) grew well and No. 357 gave birth to healthy twins. Group II (Chart I), which received orange juice treated with hydrogen peroxide at room temperature, did not grow quite as well as those in Group I and they were in much poorer physical condition, tending to softness or flabbiness. Animals 366 and 367 also gave birth to twins but the young were born dead in both instances. Comparison of the animals in Groups III and IV (Chart II) shows that pasteurization of the orange juice (Group III) had no detrimental effect, for the growth curves are as good as those which received the unheated raw orange juice (Group I).

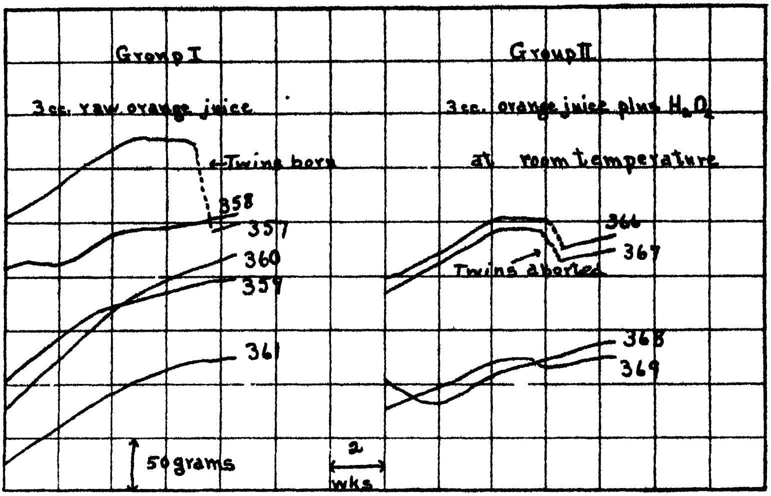

Chart I.

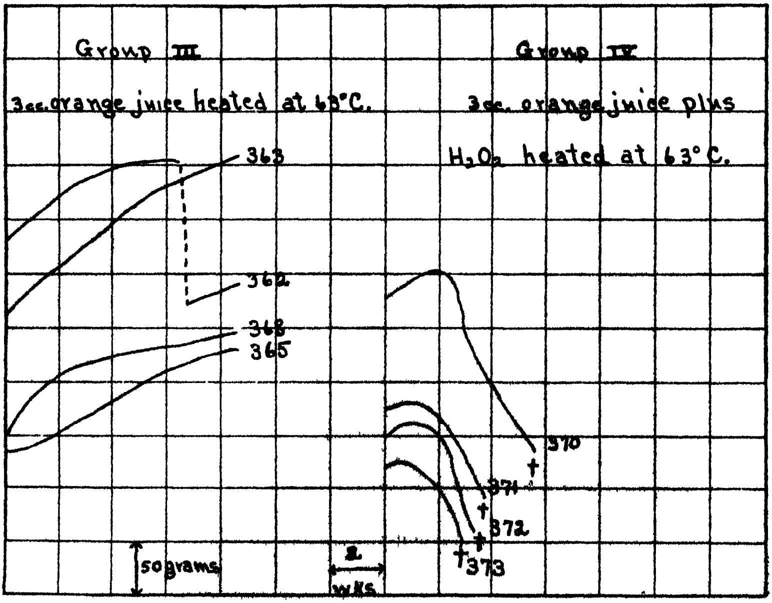

Chart II.

It would appear, therefore, that heat alone at 63°C. does not destroy the antiscorbutic vitamine. All the animals in Group IV, which had received orange juice that had been treated with hydrogen peroxide at 63°C., died with pronounced scurvy symptoms and lesions. These animals died within 3 to 6 weeks (average 4 weeks), while those in Group III were in excellent condition at the end of 2 months. Animal 362 (Group III) also gave birth to healthy twins, indicating that pasteurization had no harmful effect.

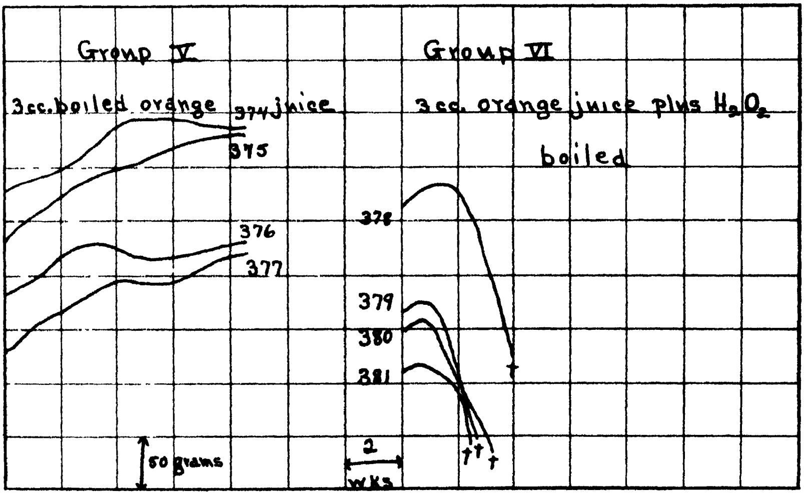

Chart III.

The growth curves of the animals in Group V (Chart III) indicate that boiling for 30 minutes has no destructive action upon the antiscorbutic vitamine, for all of the animals were in excellent physical condition at the end of the experimental period. The influence of hydrogen peroxide during the boiling process, however, is very marked. All the animals of Group VI (Chart III) died with scurvy in less than 30 days. This would indicate that the speed of the oxidative destruction of the vitamine is hastened as the temperature increases, appearing to conform to the recognized chemical laws regarding the influence of heat on chemical reactions.

SUMMARY AND CONCLUSIONS.

1. The antiscorbutic vitamine is not destroyed by heating at pasteurization temperature (63°C.) for 30 minutes in closed vessels or by boiling (100°C.) for 30 minutes under reflux condensers.

2. Hydrogen peroxide possesses some destructive action when added to orange juice at room temperature and the destructive action is increased when the orange juice-hydrogen peroxide mixture is heated at 63 and 100°C.

3. The antiscorbutic properties of orange juice are susceptible to oxidation but, in the absence of oxidizing agents, are stable to heat up to the boiling temperature of orange juice.

A SIMPLE LABORATORY GAS METER AND AN IMPROVED HALDANE GAS ANALYSIS APPARATUS.

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Carpenter (1) has described in detail the methods which are available for the study of respiratory exchange in man. Of these the simplest is that of Douglas which depends upon the collection of a specimen of expired air in a rubber bag and the determination of its CO_2 and O_2 percentages in a Haldane gas analysis apparatus (2). Since the publication of Carpenter's work, several types of apparatus have appeared. They all determine the caloric production by methods of measuring the reduction in the oxygen volume in a closed system which includes the patient's respiratory tract. The methods, while simple, are not always satisfactory. In so far as they are limited to the determination of oxygen consumption, they fail to give as much information as is frequently desired concerning the gaseous metabolism. This paper describes some improvements in apparatus to be used in connection with methods similar to those of Douglas.

For the collection of expired air, one may use in addition to a suitable face mask and valves, either a calibrated spirometer (3) or a properly constructed rubber bag. A spirometer is less portable and more expensive. The rubber bag designed by Douglas furnishes an easy and portable means of collecting the expired air. For the measurement of the volume of its contents, it is customary to use a wet meter of the Bohr type. The Danish meters are very expensive, but those made in this country, while less elegant, are very reliable. They maintain vapor saturation of the expired air but there is no data available as to the error which they introduce by the absorption of the CO_2 from the air passed. They are not very portable and to read correctly must have the water level closely adjusted. In the case of the $\frac{1}{10}$ cubic foot size, the volume read is 1 per cent low for each $\frac{1}{16}$ inch rise of the water level above the mark set. The meters have a slow rate of flow and if the rate be more than about $\frac{1}{3}$ cubic foot per minute there results a rise in the water level producing a contraction in the volume reading which may readily amount to 4 per cent. It is easy by increasing the rate, to cause the air to bubble through the meter and increase this error to 20 per cent or more.

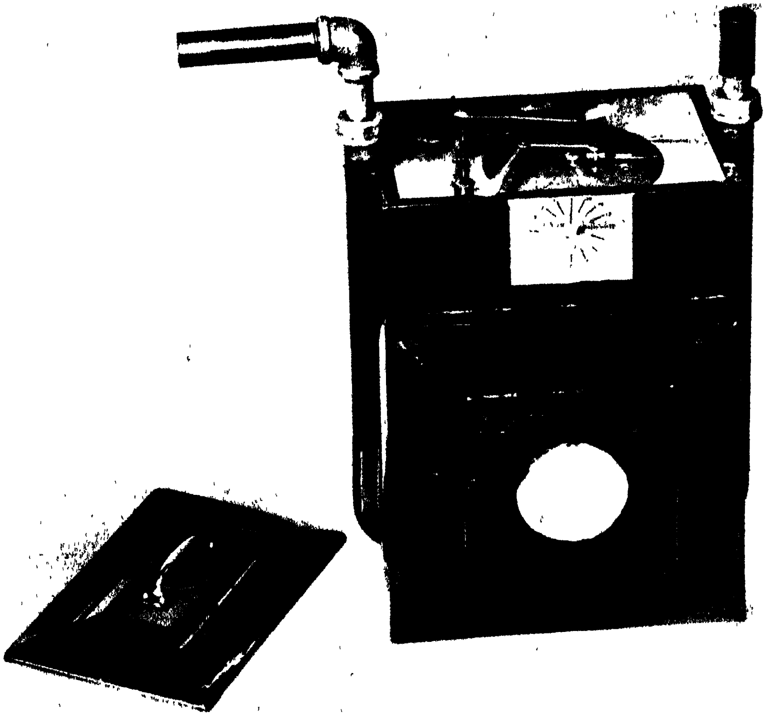

Fig. 1.

An inexpensive and portable meter may be adapted from the ordinary five light gas service meter (Fig. 1). This meter has two soft oiled leather bellows in closed compartments, and works as a tandem reciprocating engine to rotate a vertical shaft at the rate of one revolution per $\frac{1}{8}$ cubic foot of gas passed. A worm drive from this operates the dial. The top of the meter may be removed, or the meter purchased without a top, and a graduated circle and hand placed on the vertical shaft. It then reads about 3,540 cc. per revolution. The factory adjustment of the meter is made to within about 1.0 per cent of this figure. The meter may be calibrated by displacing air from a large bottle with tap water.

The calibration of two such meters was done by delivering air from a 10 cubic foot standard meter prover of the spirometer type in one of the laboratories of the United Gas Improvement Company. The air was delivered under pressures varying from 0.5 to to 2.5 mm. of Hg. The speed varied from 2 to 4 cubic feet a minute. A test performed with a standard cubic foot bottle, correct to one part in ten thousand and delivering air much slower, gave the same readings as the slowest of the above speeds. During numerous consecutive runs the variation in the meter reading was at any one pressure never more than two parts in one thousand. There was a variation due to pressure changes. Up to a speed of 2 cubic feet per minute the readings were constant. At 3 cubic feet per minute (1.6 mm. of Hg) the reading for 10 cubic feet (corrected) was 0.6 per cent low and at 4 cubic feet per minute (2.5 mm. of Hg) the reading was 1.0 per cent low. When calibrated at low speeds and used at higher speeds the meter reading can therefore be increased in these proportions. The decreased readings at higher pressures do not seem to be due to leakage through the reciprocating valves of the meter.

If the zero of the dial on the main shaft be taken as the top with the meter facing the operator, then Fig. 2 represents the calibration curves of two of these meters when run in series with a Bohr meter. The hand does not run evenly around the circle but varies by as much as 180 cc. from the expected value. The correction curves for the two instruments are about alike and the unevenness is a part of the design of the apparatus. A calibration curve of this form will correct for this error and such a correction may be applied in case the meter is used under conditions in which such a difference is of importance. For metabolism experiments in which the total volume is large compared with 180 cc., this error may be neglected. It is of about the order of magnitude of the error in reading the position of a spirometer bell.

The expired air is saturated with water vapor and is at a relatively high temperature. If it is, however, allowed to cool before being measured care must be taken that the meter is not warmer than the air for in passing through it will be warmed and no longer be vapor-saturated. To obviate this, on admitting the air to the meter, it may be passed through a water bottle slightly warmer than the meter. The temperature of the air whose volume is measured by the meter had best be taken by hanging a thermometer in the exit tube. The thermometer should reach its end-point quickly.

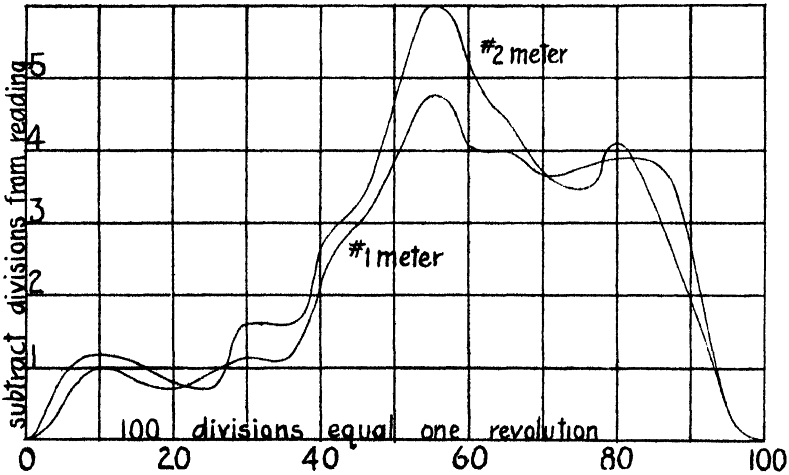

Fig. 2.

For the analysis of the expired air it is customary to use the portable gas analysis apparatus devised by Haldane (Fig. 3). The construction of this, as it is ordinarily obtained, is crude. It may be much improved by constructing a more adequate rack for the mercury leveling bulb and adding a similar one for the potash bulb. The various parts are usually connected by pieces of rubber tubing. These dry out and in any event are subject to leaks which are always suspected as possible sources of error. The principal glass parts may be blown in one piece so that there is only connecting rubber tubing below liquid levels. It is advisable to place a mercury trap between the burette and potash bulb, a feature which frequently saves much time. An additional improvement is to place all the glass parts in a water bath instead of only the burette and its control. The apparatus is then less sensitive to currents of air producing temperature changes. Since it may be desirable to fill the gas burette to exactly 10 cc., it is necessary

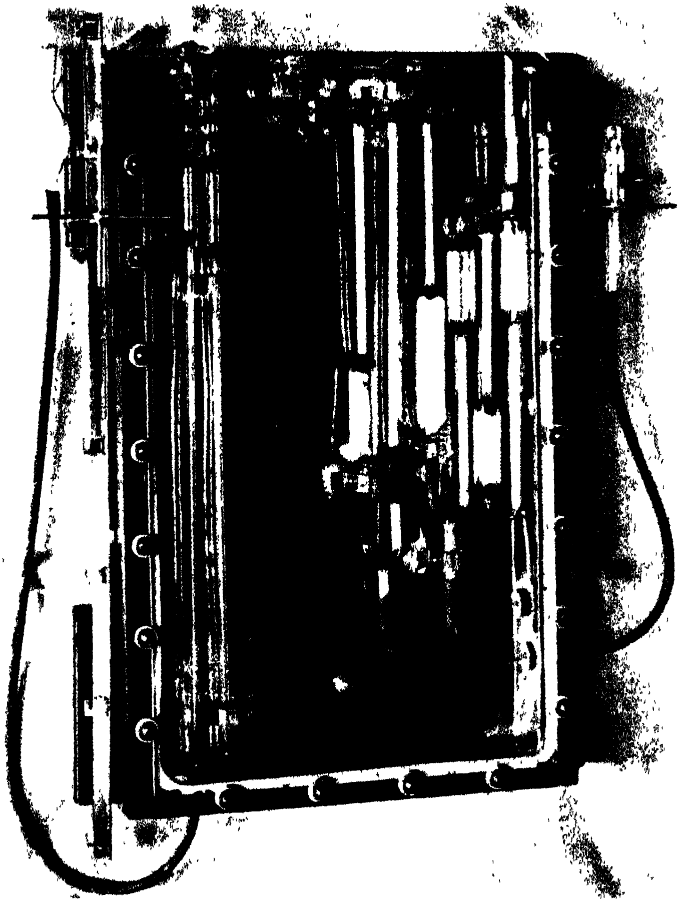

Fig. 3.

to have several graduations on the potash burette, about five at millimeter intervals, one of which may serve as an initial reading. The water bath may be constructed of a copper-lined box faced in front with plate glass, using rubber tubing as a washer between the copper flange and the glass. The two racks and pinions may then be placed one at each side of the instrument and the connections to the leveling bulbs brought out through corks inserted in thimbles in the walls of the bath. These rather simple improvements do much to make the apparatus easier to work with.[1]

SUMMARY.

There is described a simple, accurate, and relatively inexpensive laboratory gas meter, being an adaptation of the common five light service meter.

Some improvements in the Haldane gas analysis apparatus are described.

BIBLIOGRAPHY.

1. Carpenter, T. M., *Carnegie Inst. Washington, Pub. 216*, 1915, 67.
2. Haldane, J. S., Methods of air analysis, London and Philadelphia, 1912.
3. Boothby, W. M., and Sandiford, I., Laboratory manual of the technic of basal metabolic rate determinations, Philadelphia, 1920.

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[1] The meter with dials attached and the modified Haldane apparatus may be obtained from the Arthur H. Thomas Co., Philadelphia.

LIPASE STUDIES.

I. THE HYDROLYSIS OF THE ESTERS OF SOME DICARBOXYLIC ACIDS BY THE LIPASE OF THE LIVER.

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(Received for publication, May 24, 1921.)

The hydrolysis of the esters of the dicarboxylic fatty acids, malonic and succinic, by lipase has not been the subject of careful study. Doyen and Morel (1) observed that serum lipase caused hydrolysis of diethyl succinate, but the results are difficult of interpretation from a quantitative standpoint. Kastle (2) stated that diethyl succinate was readily hydrolyzed by liver lipase, but cited no quantitative data as to the extent of hydrolysis. He also observed that the metallic salts of the monoethyl esters of the dicarboxylic acids, *e.g.* sodium ethyl succinate, were not split by the lipase of the liver. He considered that the inactivity of lipase toward this class of compounds was due to the fact that they are ionized and that lipase is unable to attack ionized compounds. Morel and Terroine (3) undertook a comparative study of the hydrolysis of the diethyl esters of malonic, succinic, glutaric, suberic, and sebacic acids by the pancreatic juice of the dog, and concluded that the ease of hydrolysis increased with the increase of molecular weight of the ester up to glutaric acid. The maximum hydrolyses obtained were 2.6 per cent for ethyl malonate and 12.9 per cent for ethyl succinate. It is difficult to interpret their results inasmuch as no very definite details as to the amounts of ester and enzyme used or the dilution employed are given. It would appear, however, that the concentration of ester was such that a considerable part of it must have remained in suspension or emulsion. Moreover, the time (6 to 7 hours in most experiments) was too short to allow the reaction to reach an equilibrium, especially with the high concentration of ester in the reaction mixture. To obtain optimum conditions for the action of lipase, it is desirable that the concentration of the ester be such that complete solution is effected and that the dilution be sufficiently high to prevent inhibition of the action of the enzyme by the acid resulting from the hydrolysis of the ester.

In the experiments to be reported in the present paper the hydrolysis of diethyl malonate and diethyl succinate by the lipase of hog liver has been studied. Every attempt has been made to observe the optimum conditions for lipolytic activity in order to obtain the maximum hydrolysis possible. This has been effected by the use of dilute solutions (0.05 to 0.0125 N) and by frequent retitrations to neutralize the acidity developed which might inhibit the activity of the enzyme. The results obtained have confirmed the work of Kastle (2) on the stability of the metallic salts of the monoethyl esters of the dicarboxylic acids toward lipase. They indicate, however, that the hydrolysis of the diethyl esters studied can proceed only to the removal of one ethyl group and that the monoethyl esters as well as their metallic salts are stable toward lipase.

EXPERIMENTAL.

Preparation of Lipase.—The lipase was prepared from fresh hog liver by the use of glycerol as an extraction agent according to the method of Kanitz (4). The liver was obtained directly from the slaughter house and the extraction made within 1 hour after the death of the animal. After thorough admixture the extract was allowed to stand 2 months and then strained through cheese-cloth for use in the experiments. The use of a glycerol extract is advantageous since the glycerol not only furnishes a medium in which bacterial action is inhibited but also fails to extract fats and lipoids which by their autolysis might obscure the acidity developed by hydrolysis of the esters under investigation. Our observations are in agreement with the results of Simonds (5) who has reported that glycerol extracts of liver may retain their ester-splitting power unimpaired for some months.

The Esters.—The purity of the diethyl malonate and succinate used as substrates was checked by determinations of their boiling points and saponification values The potassium ethyl malonate was a pure white crystalline compound. The preparation of the acid ethyl malonate presented some difficulties. The procedure finally adopted was as follows. 4.25 gm. of pure potassium ethyl malonate were carefully weighed out and dissolved in a small volume of water, and the calculated amount of concentrated sulfuric acid was added. This was sufficient to convert the potassium salt to the monoethyl ester. After the addition of the acid the mixture was thoroughly extracted with twice its volume of ether and the extraction repeated twice. The combined ether extracts were then washed repeatedly with small amounts of water to remove traces of sulfates from the ether layer. After the evaporation of the ether at room temperature there remained a light-colored oily residue. This was carefully washed into a volumetric flask and made up to a liter with distilled water. No test for sulfates was obtained in this solution.

For the analysis of the monoethyl ester prepared as described 25 cc. of the solution were titrated with 0.09818 N sodium hydroxide using phenolphthalein as an indicator, and the saponification value of this neutralized solution determined in the usual manner. Results (averages of closely agreeing duplicate determinations) of the analysis of two different preparations are given.

25 cc. of solution required 4.10 cc. of 0.09818 N NaOH for the neutralization of the free acidity and 4.20 cc. of alkali for the subsequent saponification of the ethyl group.

25 cc. of the solution required 4.00 cc. of 0.09818 N NaOH for the neutralization of the free acidity and 4.20 cc. of alkali for subsequent saponification of the ethyl group.

These analytical results check satisfactorily with the theoretical for acid ethyl malonate, which would require that the alkali used for the neutralization of the free acid group should be equal to that used for the saponification of the ethyl group. It was computed also from these analyses that the normality of the solution of acid ethyl malonate was approximately 0.033 N.

Determination of the Action of the Lipase on the Esters.—All hydrolyses were carried out at room temperature. To 25 cc. of the ester (usually in 0.05 N concentration) was added 0.5 cc. of the strained glycerol extract of liver and after incubation for the desired period of time, the acidity developed was titrated with 0.09818 N sodium hydroxide with phenolphthalein as indicator.

The flasks were arranged in pairs and one pair titrated after 15 minutes, a second pair after 30 minutes, etc. Each pair of flasks was also retitrated at the intervals shown in the tables and the figures given represent the total volume of standard alkali required for neutralization. The figures presented in the tables are the averages of check determinations from which the blanks due to the acidity of the extract and of the esters have been subtracted. When ethyl propionate or butyrate has been used as substrate[1] it has been possible to obtain a hydrolysis of 85 to 90 per cent in 7 hours with the above procedure.

The significance of the tables will perhaps be made more evident by the following explanation. In Table I with 0.05 N ester, under the heading 1 hour, the figures 3.80, 3.30, 2.75, and 1.70 cc. are given. The figure 1.70 represents the average acidity developed in two flasks which were first titrated at the end of an hour. The figure 2.75 represents the average total acidity of two flasks which were first titrated at the end of a period of 45 minutes and again 15 minutes later at the end of an hour. Similarly 3.30 cc. of the standard alkali were required to neutralize the acidity in two flasks which were first titrated at the end of a period of 30 minutes, and then at 15 minute intervals at the end of periods of 45 and 60 minutes. It will be noted that the rate of hydrolysis is increased by neutralization of the acidity as it develops. Thus at the end of an hour in the case just discussed, the acidity produced was equivalent to 1.70 cc. of the standard alkali in the flasks not previously titrated, while the total acidities developed in those flasks in which 2, 3, and 4 titrations had been made during the same period were 2.75, 3.30, and 3.80 cc., respectively. This increased rate of hydrolysis continues up to the point of equilibrium and no further increase is then noted. Thus with 0.0125 N ester (Table I) the equilibrium point (1.35–1.40 cc.) is reached in 1 hour when the neutralization of acidity has been made at 15 minute intervals from the beginning of the experiment, and in 3 hours when no previous neutralization has occurred.

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[1] Unpublished data.

TABLE I.

Comparative hydrolysis of 0.05, 0.025, 0.01666, and 0.0125 N solutions of diethyl succinate by lipase of hog liver, expressed in cc. of 0.09818 N NaOH required for neutralization of acid formed. 25 cc. portions of the ester solution were employed. For complete saponification 12.73, 6.36, 4.24, and 3.18 cc. of 0.09818 N NaOH, respectively, are required.

Time 15 min	30 min	45 min	1 hr.	2 hrs	3 hrs.	5 hrs	7 hrs.	20 hrs.	Concentration of ester.
Standard NaOH required for neutralization of acidity.									
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
0.70*	2.00	3.10	3.80	5.45	5 65	5.65	5.65	5.65	
	1.20	2.70	3.30	5.55	5.75	5.75	5.75	5.75	
		1.65	2.75	4.80	5.60	5.65	5.65	5.65	
			1.70	4.75	5.60	5.60	5.60	5.60	
				2.90	5.60	5.60	5.60	5.60	0.05 N
					3.60	5.60	5.60	5.60	
						4.60	5.75	5.75	
							4.80	5.45	
								5.75	
0.55	1.45	2.10	2.45	2.70	2.70	2.75	2.75	2.75	
	0.60	1.50	1.90	2 65	2.70	2.70	2.70	2.70	
		0 85	1.35	2 60	2.65	2.65	2.65	2.65	
			0.60	2.05	2.45	2.50	2.50	2.50	
				1.65	2.65	2.65	2.65	2.65	0.025 N
					2.30	2.70	2.70	2.70	
						2.50	2.70	2.70	
							2.45	2.80	
								2.70	
0.55	1.25	1.60	1.80	1.80	1.85	1.85	1.85		
	0.75	1.35	1.75	1.75	1.75	1.85	1.85		
		1.10	1.75	1.85	1.85	1.85	1.85		
			1.10	1.70	1.70	1.80	1.80		0.01666 N
				1.70	1.95	1.95	1.95		
					1.75	1.85	1.85		
						1.70	1.85		
							1.75		

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* In all the tables, the last figure in each vertical column represents the amount of alkali required for neutralization of the acidity developed during the period represented. Each figure in the horizontal column to the right of the first figure represents this amount of alkali plus the additional amounts of alkali required for retitration at the intervals indicated. For further explanation of this and succeeding tables see the text.

TABLE I—*Continued.*

Time 15 min.	30 min	45 min	1 hr.	2 hrs	3 hrs.	5 hrs.	7 hrs.	20 hrs.	Concentration of ester.
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
0 30	0.80	1.15	1.35	1.40	1.40	1.45	1.45		
	0 50	1.00	1.30	1.35	1.35	1.40	1.45		
		0.65	1.15	1.40	1.40	1.40	1.40		
			0.80	1.45	1.45	1.45	1.45		0.0125 N
				1.20	1.45	1.45	1.45		
					1.40	1.45	1.45		
						1.40	1.50		
							1.45		

DISCUSSION.

In Table I are presented the data obtained from the hydrolysis of solutions of diethyl succinate in concentrations ranging from 0.05 to 0.0125 N by the lipase of the liver. It will be noted that regardless of the dilution of ester employed in no case was the hydrolysis greater than 50 per cent of the theoretical. Since with the esters of the monocarboxylic fatty acids (*e.g.*, ethyl propionate) hydrolysis was nearly complete under the same conditions this seemed to indicate either that at this stage equilibrium had been reached in the reaction or that the reaction was more nearly complete than appeared and that definite products were formed other than succinic acid and ethyl alcohol. Further consideration of the results showed that the percentage of hydrolysis in all the various dilutions closely approximated 90 per cent of the theoretical provided only one ethyl group was removed from the diethyl ester by the action of the lipase. The fact that equilibrium was reached at approximately the same point in each dilution would further indicate that a condition of equilibrium between diethyl succinate on the one hand and ethyl alcohol and succinic acid on the other at the point where about 45 per cent hydrolysis had occurred had not been reached. If the reaction were as incomplete as this would indicate, change in the dilution of the substrate should alter the station of equilibrium and the percentage hydrolysis increase with increasing dilution. Thus Taylor (6) has shown in his studies on the action of the lipase of the castor bean upon triacetin that under like conditions a variation in the concentration of ester from 0.5 to 2.0 per cent resulted in a lowering of the percentage of ester hydrolyzed from 86 to 70. Kastle, Johnston, and Elvove (7) have also maintained that the more dilute the solution the greater the rate of lipolysis. In the present experiments the equilibrium corresponding to about 90 per cent hydrolysis of diethyl to monoethyl succinate was reached in the higher dilutions in 2 to 3 hours and remained unchanged thereafter.

The results of the experiments with diethyl malonate as substrate (Table II) are also in harmony with the theory that hydrolysis of the diethyl ester proceeds only to the removal of one ethyl group. The hydrolysis progressed rapidly to the point which corresponded to 85 to 90 per cent cleavage of one ethyl group and remained nearly constant thereafter.

TABLE II.

Hydrolysis of 0.05 N diethyl malonate by lipase. 25 cc. of 0.05 N solution of ester were used. For the complete saponification of this amount of ester 12.73 cc. of 0.09818 N NaOH are required.

Time 15 min	30 min.	45 min	1 hr.	2 hrs	3 hrs	4 hrs	5 hrs	7 hrs.
Standard NaOH required for neutralization of acidity.								
cc.	cc	cc	cc.	cc.	cc.	cc.	cc.	cc
0.65	1.25	2.05	3.05	3.85	5.05	5.65	5.95	6.05
	0.65	1.30	2.35	3.05	4.45	5.30	5.75	6.05
		1.00	2.15	2.95	4.50	5.30	5.80	6.00
			1.40	2.25	3.85	4.80	5.40	5.80
				1.60	3.30	4.30	5.10	5.80
					2.75	3.80	4.70	5.65
						3.30	4.35	5.60
							3.75	5.05
								4.00

In order to test the hypothesis that the second ethyl group of the diethyl ester could not be readily hydrolyzed by the lipase a study was made of the action of lipase on the acid ethyl malonate (Table III). No appreciable hydrolysis occurred in 24 hours. In order to determine whether this inactivity of the lipase was due to the inhibitory influence of the free acid group the action of lipase on potassium ethyl malonate was also investigated (Table IV). In confirmation of the work of Kastle (2) on sodium ethyl succinate and other salts of monoethyl esters, no hydrolysis was observed. Inasmuch as both the acid ethyl ester and its potassium salt had been demonstrated to be stable toward the hydrolytic action of the lipase, a series of experiments was carried out to determine whether the acidity and the presence of potassium ions in the compound were the factors which inhibited or destroyed the lipase. Solutions were prepared containing the same concentration of the potassium and acid ethyl malonates as used in the previous experiments and ethyl propionate added to each. The action of lipase on these 0.05 N solutions of ethyl propionate in the presence of potassium and acid ethyl malonates is presented in Table V. The potassium salt of the ester exerted no demon-

TABLE III.

The action of lipase on acid ethyl malonate. (See text for the preparation of the acid tester.) 25 cc. portions were used. Initial acidity due to the acid group of ester plus the acidity of the glycerol extract is equivalent to 4.30 cc. of 0.09818 N NaOH. This blank has not been deducted.

Time 30 min.	1 hr.	3 hrs	4 hrs	5 hrs	7 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.						
cc.	cc.	cc.	cc.	cc.	cc.	cc.
4.35	4.35 4.30	4.35 4.30 4.25	4.35 4.30 4.25 4.30	4.35 4.30 4.25 4.35 4.30	4.35 4.30 4.25 4.35 4.30 4.30	4.55 4.65 4.50 4.55 4.55 4.50

TABLE IV.

The action of lipase on potassium ethyl malonate. 25 cc. of 0.05 N solution were used.

Time ....30 min	1 hr.	2 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.			
cc.	cc.	cc.	cc.
0.00	0.00 0.05	0.00 0.05 0.05	0.10 0.15 0.15 0.05

strable influence on the course of the hydrolysis, the reaction proceeding at the usual rate. When acid ethyl malonate was present in the solution, the results were otherwise. No appreciable splitting of the easily hydrolyzed ethyl propionate occurred in 4 hours and little in 24. These experiments would indicate that the acidity of the monoethyl ester of the dicarboxylic acids studied may be one factor which inhibits the action of lipase on this type of compounds.

TABLE V.

The action of lipase on 0.05 N solutions of ethyl propionate in the presence of acid ethyl malonate and potassium ethyl malonate. 25 cc. portions were used. 12.73 cc. of 0.09818 N NaOH are required for complete saponification of this amount of ethyl propionate.

<table>
<tr><th colspan="5">0.05 N ethyl propionate and potassium ethyl malonate.</th></tr>
<tr><th>Time 2 hrs.</th><th>4 hrs.</th><th>12 hrs</th><th>17 hrs.</th><th>24 hrs.</th></tr>
<tr><td colspan="5">Standard NaOH required for neutralization of acidity.</td></tr>
<tr><td>cc.</td><td>cc.</td><td>cc.</td><td>cc.</td><td>cc</td></tr>
<tr><td>3.30</td><td>6.25</td><td></td><td>7.80</td><td></td></tr>
<tr><td></td><td>4.00</td><td></td><td>8.15</td><td></td></tr>
<tr><td></td><td></td><td>6.95</td><td>9.55</td><td></td></tr>
<tr><td colspan="5">0.05 N ethyl propionate and acid ethyl malonate.[*]</td></tr>
<tr><td>0.45</td><td>2.35</td><td></td><td></td><td></td></tr>
<tr><td></td><td>0.50</td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td>2.30</td></tr>
</table>

[*] The blank due to initial acidity of acid ethyl malonate has been subtracted.

Since all the experimental evidence thus far obtained indicated that the lipase of the liver could split the diethyl esters of the series under investigation only to the monoethyl esters, an attempt was made to isolate and identify the monoethyl derivative among the products of the lipolytic action 3.75 cc. of ethyl malonate were added to water (100 cc.) with 5 cc. of the glycerol extract, and hydrolysis was allowed to proceed with frequent neutralization of the acidity by 0.09818 N sodium hydroxide, until the acidity developed over a period of several hours became almost negligible. For complete hyrolysis of this amount of ester, 506 cc. of the standard alkali should have been required, but it was found that even with frequent retitrations the reaction reached an equilibrium when about 240 cc. of alkali had been added, a figure which is in rather striking confirmation of the experimental results obtained previously with smaller amounts of ester. The reaction mixture was then filtered and carefully extracted three times with ether. This should have removed any unchanged diethyl malonate which would have been present in considerable amount if malonic acid and ethyl alcohol had been the products and the reaction had reached an equilibrium at a hydrolysis of less than 50 per cent as indicated by the titration figures. If on the other hand, the products formed were acid ethyl malonate and ethyl alcohol, the amount of unchanged diethyl malonate should have been very slight. The ether extract was carefully evaporated at room temperature, and the residue dissolved in water. Aliquots of this solution contained no material which could be saponified either by alkali or by the glycerol extract of hog liver. This furnished evidence that no significant amounts of diethyl malonate remained after hydrolysis by lipase.

The solution which remained after the ether extraction was acidified with sulfuric acid and repeatedly extracted with ether. The combined ether extracts were washed several times with water to remove traces of sulfates and sulfuric acid, and the ether removed by evaporation at room temperature. The oily residue of a light yellow color was dissolved in water and made up to 200 cc. No sulfates were detected in this solution. Quantitative tests were carried out on aliquots in the manner similar to that outlined in another part of this paper for the analysis of the acid ethyl malonate prepared from potassium ethyl malonate. If the chief product of the reaction had been malonic acid, then no saponifiable material should have been present after neutralization of the free acid. If ethyl acid malonate had been formed, however, the alkali required for neutralization of the free acid and that for the subsequent saponification of the ethyl group should have been the same. Analysis showed that 25 cc. of the preparation described required 16.30 cc. of 0.09818 N sodium hydroxide for neutralization of the free acidity and 17.43 cc. for the subsequent saponification of the neutralized aliquot. Analyses of the products of another experiment carried out under similar conditions showed titrations of 16.25 and 18.17 cc., respectively. When the possible sources of error in an experiment of this sort are considered, these results may be considered satisfactory and to offer further proof in support of the theory that the monoethyl ester is the main product of the hydrolysis of the diethyl esters of succinic and malonic acids by the lipase of hog liver and that the monoethyl ester is hydrolyzed with difficulty, if at all. Further studies to determine whether lipases from other sources behave similarly are in progress.

SUMMARY.

On the basis of the acidity developed when the lipase of hog liver was allowed to act upon the diethyl esters of succinc and malonic acids, it is considered that the reaction proceeded to an equilibrium which corresponded to the removal of one ethyl group from the diethyl esters. A substance was obtained from the products of the reaction between diethyl malonate and lipase which gave on analysis figures which were in good agreement with those required for monoethyl malonate. Lipase of hog liver was not able to effect the cleavage of monoethyl malonate or potassium ethyl malonate. Ethyl propionate was hydrolyzed by lipase in the presence of the potassium salt of monoethyl malonate, but not in the presence of the monoethyl ester itself.

BIBLIOGRAPHY.

1. Doyen, M., and Morel, A., *Compt. rend. Soc. biol.*, 1903, lv, 682.
2. Kastle, J. H., *Am. Chem. J.*, 1902, xxvii, 481.
3. Morel, L., and Terroine, E., *J. physiol. et path. gén.*, 1912, xiv, 58.
4. Kanitz, A., *Z. physiol. Chem.*, 1905–06, xlvi, 482.
5. Simonds, J. P., *Am. J. Physiol.*, 1919, xlviii, 141.
6. Taylor, A. E., *J. Biol. Chem.*, 1906–07, ii, 87.
7. Kastle, J. H., Johnston, M. E., and Elvove, E., *Am. Chem. J.*, 1904, xxxi, 521.

STUDIES ON EXPERIMENTAL RICKETS.

VIII. THE PRODUCTION OF RICKETS BY DIETS LOW IN PHOSPHORUS AND FAT-SOLUBLE A.

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Plates 4 to 7.

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INTRODUCTION.

In a brief preliminary report[1] we described a faulty ration and its effects on the skeleton of the growing rat, which corresponded in all essential details to rickets as that disease manifests itself in the skeleton of the human being. The faulty ration in question, No. 3127, was deficient in phosphorus and fat-soluble A.[2] In other respects it was, apparently, satisfactorily constituted. It contained protein of good quality and adequate in amount. The calcium was furnished in the form of the carbonate in a proportion (2 per cent of the total ration) which was essentially the amount necessary for the best promotion of growth, longevity, and reproduction. We took pains to point out that, when the content of phosphorus in the faulty ration was raised through the substitution of 2.5 per cent $CaHPO_4$ for the 2 per cent $CaCO_3$, osteoporosis and not rickets developed. We also called attention to the fact that, when a deficiency in calcium as well as in phosphorus was created through the omission of the $CaCO_3$ from the faulty ration, a pathological condition developed in the skeleton which was entirely distinct from rickets. We showed further that, when cod liver oil was added to the faulty ration in an amount equal to 2 per cent by weight of the ration, the pathological condition which developed in the skeleton was osteoporosis and had no resemblance whatsoever to rickets. We believe the results obtained in the experiments cited to be of considerable significance. In the first place, the experiments showed that it was possible to produce a pathological condition in the rat unquestionably similar to the rickets of the human being through the diet alone. In the second place, they showed that rickets could be induced by means of a ration, the faults of which were clearly defined and sharply limited; *viz.*, deficiencies in phosphorus and fat-soluble A. In the third place, they indicate that deficiency of phosphorus in the ration insufficiently supplied with fat-soluble A would give rise to rickets only when calcium was present in a ratio considerably higher than the calcium-phosphate ratio which is optimal for ossification. The experiments indicated, therefore, that the development of rickets in this instance depended on the existence in the faulty ration of (1) a specific disproportion in the calcium-phosphate ratio, the phosphorus being low, the calcium, relatively speaking, high, and (2) an insufficiency of an organic substance contained in cod liver oil having a profound influence on the calcification of cartilage and the ossification of bone.

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[1] Read before the American Pediatric Society, Swampscott, Mass., June 3, 1921.

[2] We are at present unwilling to commit ourselves to the view that the antixerophthalmic factor and the factor concerned in the ossification and growth of the skeleton are distinct, though we are prepared to acknowledge that such may be the case. We, therefore, use fat-soluble A to describe both the antixerophthlamic and antirachitic factors, assuming that the latter has a separate existence.

In the present paper we wish to describe further the composition of the faulty ration which produced the rachitic lesions above referred to, and to present in detail the anatomical evidence on which the statement of the production of rickets was based. We also wish to describe two other similarly constituted diets, one being the diet just referred to only slightly modified, and their effects on the growth and ossification of the skeleton in the rat.

Ration 3127.

	per cent
Rolled oats	[illegible]
Gelatin	10.0
Wheat gluten	7.0
NaCl	1.0
KCl	1.0
$CaCO_3$	2.0
Dextrin	39.0

This mixture was markedly deficient in phosphorus and in fat-soluble A (antixerophthalmic substance) but was otherwise well constituted. Its proteins were abundant and of good quality; its content of calcium not far from the optimum. So far as we can judge from experimental data available, the content of other inorganic constituents was satisfactory. Whileit did not furnish much above the actual requirement of the antineuritic substance, this factor could not be regarded as influencing in any way the well being of the animals.

TABLE I.

Data Concerning Rats of Lot 3127.

No of rat	Age when put on diet	Weight when put on diet	Days on diet.	Age at death.	Xerophthalmia	Sex.	Weight.
	days	*gm.*		*days*			*gm.*
642	25	41*	24	49	+	♀	41
688	25	41	35	60	+	♂	48
689	25	41	35	60	+	♀	46
690	25	41	35	60	+	♂	46
708	25	41	39	64	+	♂	48

*The entire litter of rats composing Lot 3127 was weighed together. Each recorded weight in the column does not represent the exact weight of the individual but one-sixth the total weight of the litter. The sixth rat was partly eaten after being on the diet 35 days.

Ration 3133 was closely similar to Ration 3127 except that the former contained 0.5 per cent of butter fat in place of an equivalent amount of dextrin. This supplied a small amount of the fat-soluble A (antixerophthalmic substance) but not sufficient to entirely protect the animals against ophthalmia. This addition of butter fat was not sufficient to enable them to grow, although more liberal amounts would have done so. We know from other experiments, however, that they could not have been normal or have developed normal bones even with very liberal amounts of butter fat. The 0.5 per cent of butter fat added to their diet extended the lives of the animals to some extent.

TABLE II.

Data Concerning Rats of Lot 3133.

No. of rat	Age when put on diet.	Weight when put on diet.	Days on diet.	Age at death.	Xerophthalmia.	Sex.	Weight.
	days	*gm*		*days*			*gm.*
590	16	29*	35	51		♀	35
639	16	29	43	59		♀	27
706	16	29	59	75		♂	67
717	16	29	61	77		♀	45
811	16	29	91	107		♂	60
860	16	29	98	114		♂	39

*The entire litter of rats composing Lot 3133 was weighed together. Each recorded weight in the column does not represent the exact weight of the individual but one-eighth the total weight of the litter. Two rats disappeared from the cage; they were probably eaten.

Ration 3133.

	per cent
Rolled oats	40.0
Gelatin	10.0
Wheat gluten	7.0
NaCl	1.0
KCl	1.0
Dextrin	38.5
$CaCO_3$	2.0
Butter fat	0.5

Ration 3143.

	per cent
Wheat	33.0
Maize	33.0
Gelatin	15.0
Wheat gluten	15.0
NaCl	1.0
$CaCO_3$	3.0

This diet contains nearly twice the optimal content of calcium and is decidedly below the optimum in its content of phosphorus and of fat-soluble A. Otherwise it is well constituted. On this food mixture young rats grow somewhat more than on the other diets described in the paper. They do not develop xerophthalmia but become very deformed and die early.

When placed upon Ration 3127 the young rat lives for not more than 5 to 8 weeks. The growth momentum derived from the normal diet results in a preliminary gain. This soon ceases, however. The weight then remains stationary and declines toward the end of the experiment. The rats of Lot 3127 were 25 days old when confined to the faulty ration, and were killed after periods varying from 24 to 39 days.

TABLE III.

Data Concerning Rats of Lot 3143.

No. of rat	Age when put on diet.	Weight when put on diet	Days on diet.	Age at death.	Xerophthalmia	Sex.	Weight.
	days	*gm.*		*days*			*gm.*
571	50	64	50	100		♂	81
657	50	65	63	113		♀	82
658	50	67	63	113		♀	82
831	45	76	43	88		♂	62
879	40	60	60	100		♂	65
914	40	55	65	105		♂	68
915	40	53	65	105		♂	75
918	40	60	76	116		♀	60
919	28	56	69	97		♀	63

At autopsy the bodies were small and greatly emaciated. Almost no fat was present. The eyes were inflamed. The incisor teeth were brittle. The thorax was flattened at the sides, and in one or two animals was hollowed slightly along the line of junction of the cartilages and the ribs. In none of the animals, however, was it greatly deformed. The costochondral junctions themselves formed fusiform enlargements, most evident on the internal surface of the thorax. The enlargements were thrown into especial prominence if the thoracic wall was bent inwards. Fractures of the shaft of the ribs with callous formation situated usually not far behind the costochondral junctions were present in three of the animals. The lower end of the radius and ulna and the adjacent ends of the femur and tibia formed enlargements similar to those seen in the rachitis of human beings. When stripped of their muscles the ends of all the long bones were found to be enlarged. Fractures of the long bones of the extremities were not noted. The strength of the tibia and femur was greatly reduced and both these bones and the ribs offered little resistance when cut. Examination of the cut surface of the femur or tibia showed that the junction of cartilage and shaft was not clearly defined and that below the cartilage there lay a pale yellow zone of not very great depth which proved when examined under the microscope to be a transitional zone containing a mixture of elements derived from cartilage and shaft. Examination of the cut surface further showed the cortex to be extremely thin and the bone marrow to have approximately normal color.

The following changes were noted when the bones were examined under the microscope.[3] The epiphyseal cartilage separating the nucleus of ossification from the shaft was in most places broader than in the healthy animal and at several points was continued in irregular blue-staining prolongations toward or actually to the shaft. Its diaphyseal border did not end abruptly on coming in contact with the elements derived from the diaphysis but seemed to undergo a gradual transition into the latter. The zone of undifferentiated cartilage was represented by few cells. Almost at their beginning, close to the nucleus of ossification, the cells of the epiphyseal cartilage became arranged in columns or fascicles separated by rather more matrix than is seen in the bones of young rats on normal diets. Near their points of origin the cells composing the columns were flattened as if compressed in the long direction of the shaft and lay close to each other, but at a short distance from their points of origin became oval, round, or cuboidal. As soon as the cells of the epiphyseal cartilage underwent the expansive change just referred to, the intercellular substance which separated the cell groups became greatly diminished, so that the columns appeared to lie close to each other. Coincidently, the cells seemed to lose to a certain degree their affinity for hematoxylin with the result that both nucleus and cell body appeared more pale. The cell membrane appeared to be unusually thick. In general the nuclei were large, round, and well preserved. The columnar arrangement of cells was at some points maintained throughout the breadth of the cartilage, but at others became irregular or entirely lost as the metaphysis was approached, so that the cells seemed to be irregularly jumbled together. Wherever the cartilage cells came in proximity to the marrow elements they lost their blue color and were pale yellow. In many places the cartilage after losing its staining reaction ended abruptly in contact with the blood vessels derived from the marrow. At other places, however, the cartilage was continuous with the cartilage which formed the bulk of the transitional zone between cartilage and shaft, the rachitic metaphysis (Fig. 3).

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[3] The technique employed in these experiments was as follows: The bones were hardened in formaldehyde. They were cut after embedment in celloidin in sections 15 to 25 mikra thick and stained with hematoxylin and eosin. Control preparations were made with the freezing microtome and stained with hematoxylin and also silver nitrate after the method of von Kossa.

The metaphysis in which the elements derived from cartilage and shaft were blended in an irregular manner was not deep. Indeed in comparison with the metaphysis in the bones of the rats on Rations 3133 and 3143, it was exceedingly shallow. It was composed of the irregular prolongations of cartilage from the main mass which retained their blue stain, and of quantities of cartilage cells which had lost their staining reaction to hematoxylin entirely and were in all stages of transition into osteoid. There were osteoid trabeculæ derived largely from cartilage and having a waxy appearance. The remains of calcified intercellular substance staining deep blue and having a configuration like the cross-section of a honeycomb were formed in the metaphysis. The detailed description of the various elements composing the metaphysis will be found in the description of the histological findings of the rat on Ration 3133 (Fig. 4).

The cortex of the shaft near the metaphysis was broad and composed of a thick interlacing network of trabeculæ having for the most part central cores of calcified material and borders of osteoid. As the middle of the bones was approached, however, the cortex became thin. The trabeculæ had a central core of calcified material but peripheral portions composed of osteoid. The osteoid zones were fairly broad. In most of them a fibrillary arrangement could be made out. The bone corpuscles of the calcified portion were small and those in the osteoid borders appeared especially small and far separated from each other. They presented a variety of shapes. Some were round, some spindle-shaped, some irregular. Moreover, they were very unevenly distributed. Some resorptive phenomena could be recognized in the calcified portions of the trabeculæ. The marrow appeared normal. The nucleus of ossification showed changes both in its cartilaginous elements and in the trabeculæ exactly analogous to those described in the epiphyseal cartilage, and in the shaft.

The rats on Ration 3133 were younger (only 16 days old) when placed upon the faulty mixture than the rats of the lot on Ration 3127, and were kept on the faulty diet for longer periods of time, on the average 63 days. The shortest period was 35 days and the longest 98 days. The rats scarcely more than maintained their initial weight on Ration 3133. They were small and poorly nourished. The eyes of most of them were inflamed. The thorax was considerably deformed. The costochondral junction was considerably enlarged. In the majority of the animals they were displaced inwards and were greatly distorted. Fractures of the ribs were present with well marked callous formation. The knees, wrists, and ankles were considerably enlarged, as were the ends of all the long bones. The femurs and tibias cut with greatly diminished resistance. On section the enlargement of the ends of the long bones was especially evident and seemed to correspond to the cartilage and to a white zone between the cartilage and bone marrow which on microscopic examination proved to be the rachitic transitional zone or metaphysis. The line of junction between the cartilage and shaft was indistinct. The shafts of the long bones were not enlarged. The spleen was not increased in size in the majority of the rats but was enlarged in some.

The microscopic changes in the femurs and the tibias of the rats on this diet resembled those described in the bones of the rats on Ration 3127, but were considerably further advanced. The epiphyseal cartilage was irregularly increased in depth and seemed to merge with the metaphysis in a manner presently to be described. The breadth of the metaphysis was almost if not quite equal to the breadth of the epiphyseal cartilage. The shaft of the bone was broad where it bordered on the metaphysis but rapidly contracted so that the shaft of the bone as a whole did not seem to be enlarged (Fig. 2).

The undifferentiated cartilage was represented by only a few scattered cells or cell clusters. Almost at their beginning the cartilage cells became arranged in short columns or rather groups of columns. These were more widely separated from each other by matrix than in the healthy animal; that is, the amount of matrix seemed to be considerably in excess of that found in the rat under normal conditions. The columns or bundles of cartilage cells just referred to took their origin at somewhat different levels. They were bullet-shaped and the ends of the columns directed toward the nucleus of ossification were for the most part pointed. The cartilage cells composing them were flat, the long dimension running from side to side. Some of them had the same thickness throughout, others were spindle- or wedge-shaped. Many of them were curved like crescents, the concave side of the crescent opening diaphysealwards. The nuclei were in some instances centrally placed, in others at the side. The nuclei were long and thin, partaking of the shape of the cell, and took a very dark stain with hematoxylin. In some cells no cytoplasm could be seen; in all, the cytoplasm if present was scant and took a paler blue stain than the cell capsule or nucleus. The cell capsule was not clearly defined and could not be easily separated from the adjacent cell capsules or surrounding matrix. In most of the bundles the arrangement of the cartilage cells was quite irregular. The cells lay one upon the other, but not in continuous columns. One row of cartilage cells would appear to interdigitate with another or a third column would seem to be interpolated between two rows of cartilage cells. The matrix in the part of this portion of the proliferative cartilage near the nucleus of ossification stained blue with hematoxylin but as the hypertrophic zone of the cartilage was approached it began to lose its blue color and appeared yellow.

As the metaphysis was approached the cells of the cartilage underwent a remarkable change. The cells forming the bundles suddenly expanded assuming globular, oval, or cuboidal shapes and at the same time began to lose their power to take the blue stain of the hematoxylin and soon ceased to take it altogether. The nuclei shared in the expansion of the cells, becoming large, round, and vesicular, and took a pale blue stain. The matrix between the cell bundles which had lost its blue color earlier than the cells themselves was stained a pale yellow after hematoxylin and eosin. As the cells increased in size, the broad zones of matrix which separated them became somewhat decreased in amount with the result that the columns lay closer to each other. In many places, however, the broad zone of matrix which separated the bundles of flat cells above continued to exist between the columns of swollen cells nearer the diaphysis. As the cartilage cells underwent the sudden enlargement and alteration in staining reaction their columnar arrangement tended to become more definite. In certain places in which the cartilage was prolonged in solid masses into the metaphysis, the cells tended to retain their affinity for hematoxylin. The morphology of the cells in these irregular prolongations will be discussed later. *Calcification of the cartilage was entirely wanting in all the animals on this diet.*

The metaphysis varied considerably in its depth, and in its general character. In the majority of the animals it was deep. In one rat, however, which weighed only 25 gm. when killed, it was shallow. In all the animals it was made up of the various elements derived from cartilage and shaft which usually compose the metaphysis of a rachitic bone intermingled in an irregular manner. There were the irregular prolongations of the epiphyseal cartilage just referred to which retained their staining reactions to hematoxylin. There was cartilage which had lost its staining reaction altogether and seemed to be in various stages of transformation into osteöid. Many blood vessels were present, filled with red blood cells and surrounded by marrow elements. There were remnants of calcified matrix and in some rats considerable amounts of fibrous tissue (Fig. 5).

In all of the rats by far the greater part of the metaphysis was composed of the cartilage described as having lost its staining reactions entirely in the process of transformation into osteoid. This cartilage appeared in the sections lightly colored as cream color or pale yellow with eosin. It had exactly the same staining reaction as the osteoid borders of the trabeculæ. These cartilage cells for the most part looked swollen and presented a great variation in size, shape, and general morphology and composed the main bulk of the metaphysis. Many were large, and round, oval, or cuboidal in shape. Some were pear-shaped and some flattened. The nuclei were for the most part large and round and stained pale blue but in some the nuclei were small and deep blue or pycnotic. The cell bodies were yellow like the surrounding matrix or were actually colorless. The capsules appeared to be exceedingly thin. They were colorless in the case of many of the cells and could be distinguished only with reduced light as a transparent circle surrounding the cell. In the case of other cells the capsules retained some of the blue stain. The cartilage cells which had thus lost their staining reaction were anything but uniform. A row of globular cells like those just described might be continuous with a group of small cells, the smallest perhaps no larger than osteoblasts with correspondingly small nuclei staining deeply with hematoxylin, or they might be continuous with cells having a flat contour like those described in the cell bundles near the epiphyseal nucleus. Cells could be found which seemed to be fading away into a substance indistinguishable from the surrounding matrix. No nuclei seemed to be present in them, or the nuclei were so faintly stained as to be scarcely visible and the existence of the cells could be recognized only by the faint outline of the cell body visible under reduced illumination. In general, the cells in the metaphysis near the cartilage tended to be disposed in columns which were continuous with the columns of the cartilage proper. On the diaphyseal side of the metaphysis, however, the columnar arrangement had very largely disappeared. The large globular forms of cartilage cell were more numerous near the epiphyseal cartilage. The small forms were especially common on the diaphyseal side. The degenerating forms, also much more frequent on the diaphyseal side, were especially numerous in the immediate neighborhood of the blood vessels. Some of the blood vessels seemed to be surrounded by matrix exactly like the osteoid covering the trabeculæ in its staining properties and almost devoid of nuclei. The amount of matrix in which the cartilage cells lay was present in much greater quantity on the diaphyseal side of the metaphysis than on the side of the epiphyseal cartilage (Fig. 7).

The cells of the cartilage which retained their staining property were for the most part those composing solid prolongations from the main mass of epiphyseal cartilage. Blood vessels did not penetrate them. The retention of their normal staining properties and a more nearly normal morphology may have been due to the fact that they happened to have been protected from the influences of the diaphyseal circulation. The cells composing these irregular prolongations, however, showed considerable morphological variation. Near the epiphyseal cartilage they were frequently indistinguishable from the large globular cells close to the bundles of flattened cells. As the diaphysis was approached, however, the cells perhaps assumed a flat shape and appearance exactly comparable to the cells forming the bundles at the beginning of the epiphyseal cartilage. Nearer the diaphysis they might again become large and round and finally they might again become flat or show evidence of partial calcification or break up into small groups composed of cells globular in form and much reduced in size, with clearly defined rather thick blue staining cell capsules and separated by an abundant matrix which had lost its staining reactions to hematoxylin altogether. Blood vessels penetrated the metaphysis from the shaft, and were found at the level at which the sudden transition in the cartilage above described took place; *i.e.*, to the level at which the cartilage cells suddenly became swollen and lost their staining properties. This fact would seem to indicate that the changes in the cartilage might be initiated as the result of circulatory influences. Blood vessels of all sizes could be seen ramifying in an irregular manner through the diaphysis. For the most part, however, they were not large. The large vessels terminating in huge tufts so frequently seen in the bones of rachitic human beings were not present. The blood vessels were filled with red blood cells and were surrounded by marrow elements. In general, it would seem that they penetrated the cartilage rather by insinuating themselves along the intercellular ground substance separating the columns than by destruction of the cartilage cells themselves. Occasionally, however, places could be found in which the red blood cells were present within the cartilage cell capsule. Evidently under the abnormal conditions induced by the diet the cartilage cells could be destroyed by the vascular elements, but in general tended to persist, undergoing a slow degeneration or change into hyaline substance or possibly a change of a metaplastic nature into osteoid or connective tissue. In general, the blood vessels had a delicate endothelial lining but groups of red blood cells could be found lying in spaces in the metaphysis without evidence of surrounding endothelium. In general, the metaphysis did not appear to be disrupted by the vascular elements to the extent frequently seen in the bones of rachitic human beings.

Though in none of the bones of the animals on this diet was there calcium deposition in the cartilage, in almost all there were irregular calcium deposits at one point or another in the metaphysis. In one animal a zone of calcium deposition extended completely across the metaphysis, bisecting it. The calcium deposits were delicate and for the most part completely surrounded the cartilage cells, having in sections an appearance like that seen in a cross-section of a honeycomb. The cartilage cells in the metaphysis encased in calcified matrix showed morphological changes and loss of staining reactions comparable to those in evidence in the cartilage of the metaphysis elsewhere. In some of the interstices of the calcified matrix red blood cells and other marrow elements could be seen.

In one or two of the rats there was considerable connective tissue formation exactly similar to the fibrous tissue commonly seen in the rickets of human beings. As the shaft was approached the numbers of the blood vessels increased and the numbers of cells of diaphyseal origin became more numerous. Some of them bordering the blood vessels had the morphology of osteoblasts, others of connective tissue cells. Trabeculæ devoid of any calcified matter but containing instead cores of cartilage cells which varied greatly in size and were in various stages of transformation or degeneration into osteoid were seen in the shaftward portion of the metaphysis. The junction of the diaphysis and metaphysis in most of the rats was marked by calcified intercellular substance indicating the level in the growing end of bone at which the effect of the faulty diet first became manifest.

The cortex was thin and the trabeculæ were covered with osteoid. The cells of the osteoid investments were for the most part elongated, resembling connective tissue cells, and were widely separated from each other. The osteoid had a fibrillar arrangement. In some of the animals there were large osteoid formations on one side of the bone or the other under the periosteum. Few evidences of resorptive activity could be found. The marrow elements appeared to be essentially normal (Fig. 6).

In experiments with the rats of Lot 3143 the normal diet was stopped and the faulty ration substituted at ages ranging from 28 to 50 days. The animals were allowed to live on the faulty ration for from 43 to 76 days. When placed upon the faulty ration they continued to gain slowly or remained stationary in weight. They never developed inflammation of the eyes (xerophthalmia) on account of the presence of sufficient protective substance in the wheat and maize of the diet. If this diet is continued over a sufficient length of time the animals die. Just before the end of their lives rats on this ration show a definite loss of control of the posterior extremities which results in a tottering gait. The trouble is progressive and finally results in a complete paralysis of the hind legs. This condition will be fully discussed in another communication.

At autopsy they were small and emaciated. The incisor teeth were loose and so brittle as to be easily fractured. The thorax was greatly deformed. Its anteroposterior diameter was greatly increased as compared with its lateral diameter and the lower part of the sternum projected forward as in the pigeon breast deformity of human beings. At the line of the costochondral junctions there were deep grooves. When the thorax was opened and the interior examined the deformity was enormous. The costochondral junctions and shafts of the ribs met at acute angles, the apices of which pointed toward the vertebral column and were not far from it. The costochondral junctions themselves were exceedingly large and some of those of the lower ribs were twisted into the most bizarre shapes. The posterior epiphyseo-diaphyseal junctions were enlarged and appeared like so many white beads on either side of the spinal column. Many fractures of the shafts of the ribs were present, marked by large white callous formations. The spinal column itself was bent in various curvatures. The wrists, knees, and ankles were greatly enlarged, as indeed were the ends of all the long bones. Even the scapulæ were deformed. Fractures of the tibia marked by angular deformity and huge callous formation were present in some of the rats at the junction of the upper third with the lower two-thirds.

The description of the microscopic changes in the bones of the animals on this diet corresponds in its main features to the description already given of the microscopic changes in the rats on

Ration 3133. We shall limit the description of the microscopic changes, therefore, to a few observations. The enlargement of the ends of the long bones was due to an increased depth and probably also to an increased breadth in the epiphyseal cartilage and to the presence of an enormous metaphysis. The width of the metaphysis was equal to that of the epiphyseal cartilage; its depth was many times greater. The groups of flattened cartilage cells constituting the columnar zone of the cartilage were very widely separated from each other by hyaline matrix. It was impossible not to think that the amount of hyaline matrix throughout the cartilage was greatly increased. The hyaline matrix early lost its power to take the hematoxylin stain. As soon as the cartilage cells had undergone the expansive change fully described in the case of the rats on Ration 3133 they rapidly lost their normal staining properties and underwent the changes of a metaplastic and degenerative character already described. By far the greater part of the metaphysis was composed of cartilage or derivatives from the cartilage. The osteoid trabeculæ of the metaphysis in the main had a cartilaginous basis; that is, in almost all of them cartilage cells in various stages of degeneration or metaplasia could be identified. The cartilage in every animal of the group was entirely free from lime salt deposits. Some irregular lime salt deposition was present in the metaphysis of certain of the animals. The blood vessels which ramified through the metaphysis were in the main small. The cortex was thin. It was composed of bone-containing scanty central cores of calcified material and with broad osteoid borders. The breadth of the osteoid borders was extreme. The bone corpuscles found in them were widely separated from each other by quantities of yellow osteoid material. The number of cells per unit of area was consequently greatly diminished. The nuclei were long, some were spindle-shaped. They resembled the nuclei of connective tissue cells much more closely than those of bone corpuscles. A fibrillar arrangement of the osteoid could be made out. Few osteoblasts or at least cells which could be identified as such could be found lining the osteoid trabeculæ. The evidences of resorption of the calcified portions of the trabeculæ were slight. The bone marrow appeared to be essentially normal (Fig. 1).

Osteoid production was extreme in the epiphyseal nuclei of ossification. The large calluses which were formed about healing fractures were composed entirely of osteoid tissue.

DISCUSSION.

The faulty rations, Nos. 3127 and 3133, containing 2 per cent added calcium carbonate, gave rise to pathological conditions in the skeleton essentially identical with those found in the human subjects of rickets. The rachitic lesions in the group of rats on Ration 3133 were more advanced than those in the group of animals on Ration 3127, presumably on account of the presence in Ration 3133 of 0.5 per cent of butter fat. This small quantity of butter fat, while insufficient to prevent the development of xerophthalmia, was sufficient to delay its advent and to diminish its rate of progress. It increased the duration of life and made possible, presumably, increased growth of the skeleton. The small amount of butter fat in Ration 3133 probably intensified the rickets-producing properties of the diet through its stimulating effect on growth and its favorable influence on the duration of life. We have already called attention to the fact that butter fat as compared with cod liver oil is exceedingly low in its rickets-inhibiting properties.[4]

Ration 3143 was derived in part from wheat and maize, which contained a certain amount of antixerophthalmic substance (more than was present in Ration 3133, which contained 0.5 per cent butter fat). Ration 3143 further differed from Rations 3127 and 3133 in having a higher calcium-phosphate ratio than either. It gave rise to the most extreme degree of rickets. The osteoid production and the metaplastic and degenerative changes in the cartilage exceeded anything ever seen in the bones of rachitic human beings. We have repeatedly observed that, if calcium carbonate in large quantities (3 to 6 per cent of the total ration) is added to a ration insufficiently supplied with the organic factor and only slightly or not at all deficient in its content of phosphorus, most pronounced changes occur at the growing ends of the long bones. The cartilage undergoes the degenerative and metaplastic changes already described, and fails to take up calcium phosphate with any regularity, or to take it up at all. The trabeculæ near the end of the shaft become surrounded with enormous quantities of osteoid and great quantities of osteoid trabeculæ largely derived from the cartilage develop between the cartilage and the shaft. The exaggerated character of the rachitic lesion brought about by Ration 3143 is explicable on the ground that the phosphate is extremely low and the calcium high (3 per cent of the total ration in the form of calcium carbonate), so that the calcium-phosphate ratio was exceedingly high. The quantity of the antixerophthalmic substance contained in Ration 3143 was sufficient to prevent effectually the development of xerophthalmia. The quantity of the antixerophthalmic substance contained in the diet, however, was insufficient to exert, or incapable of exerting, any visible inhibitory influence on the development of the rachitic lesions in the skeleton. Indeed, it seems not unlikely that the amount of the organic factor contained in the wheat and maize of Ration 3143 may have intensified the development of the rachitic changes by promoting the growth of the skeleton and increasing the duration of life of the animal, as did the small amount of butter fat in Ration 3133.

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[4] McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii.

In a preliminary communication[5] Sherman and Pappenheimer described the production of rickets in rats by means of diets containing patent flour, 95 per cent; calcium lactate, 3 per cent; and sodium chloride, 2 per cent, with and without the addition of 0.1 per cent ferric citrate. They also described the prevention of the rickets when potassium phosphate in amounts equaling 0.4 per cent of the total was added. They also observed that, when no calcium was added to their faulty rations, osteoporosis instead of rickets developed. Patent flour is one of the most deficient foods which enters into the human diet, being exceeded in this respect only by isolated foods such as starch, sugars, fats, or polished rice. Bolted flour is rather poor in protein and this is of rather poor quality. It is very deficient in calcium, phosphorus, sodium, chlorine, iron, and possibly also in potassium. The only essential inorganic element which it probably contains in amount sufficient to meet the physiological needs of an animal is magnesium. Bolted flour is also very deficient in the antineuritic substance (water-soluble B) as shown by experiments on animals and by the frequent occurrence of beri-beri in man in Labrador and Newfoundland, where bread from this source is a principle article of food. It is exceedingly poor in fat-soluble A, and in the organic antirachitic factor. The lack of antiscorbutic substance in flour is, we believe, a matter of little or no importance in the nutrition of the rat, since this species is capable of synthesizing this complex. The ration employed by Sherman and Pappenheimer was, therefore, deficient in fat-soluble A, water-soluble B, its protein content, potassium, phosphate, and water-soluble C. In the presence of so considerable a number of defects in the ration it was impossible to be sure which were operative in the production of the disease. In the light of our experience it seems probable that their results should be interpreted as follows: The development of the rachitic condition in their animals was due (1) to a disproportion in the calcium-phosphate ratio, the calcium being present in optimal proportion or in a proportion not far from the optimal (3 per cent of the *lactate* of calcium) and the phosphorus at the lowest possible level, and (2) to a deficiency of the preventive organic substance. When neither calcium nor phosphate was added, the ratio between these (in the patent wheat flour) was more nearly the optimum than after the calcium addition, and, as was to be expected, osteoporosis, not rickets, developed. The animals needed (among other things) calcium very badly; yet we witness here the apparent paradox that meeting a physiological need and without exceeding it (*i.e.* by adding to the diet 3 per cent calcium lactate) more damage of a particular kind is done than would have resulted from permitting the pronounced calcium starvation in addition to the other deficiences of their experimental diet to continue. *Apparently in the rat the profound disturbances in the deposition of lime salts in cartilage and bone and the changes in the cells of those tissues which give rise to the pathological complex known as rickets may be produced by disturbances in the diet of the optimal ratio between calcium and phosphorus in the absence of an amount of an organic substance contained in cod liver oil sufficient to prevent them. It would seem from the results of a large number of experiments, which will be published in detail soon, that in so far as calcium and phosphate are con-*

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[5] Sherman, H. C., and Pappenheimer, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 193.

cerned, the physiological relation in the diet between the two is of infinitely greater importance in insuring normal calcification than the absolute amount of the salts themselves.

In examining the microscopic preparations from the rats which served as the material for the experiments reported in this paper, we were again struck with the similarity of behavior of the cartilage cell and of the bone corpuscle under the abnormal conditions imposed by the faulty diets. On coming into direct contact with the blood vessels the cartilage cell tended to undergo a gradual degeneration or transformation into a homogeneous substance indistinguishable except in its lack of a fibrillar arrangement from the osteoid zone surrounding the calcified trabeculæ. On coming into less direct contact with the vascular elements the cartilage cell tended to revert to its original state in the undifferentiated cartilage, or to some intermediary state of development, or to undergo metaplasia into bone corpuscles, at least such as are found in the osteoid, or into connective tissue. In the process of reversion or of metaplasia of the cartilage cell in which it underwent a diminution in size, lost its characteristic staining reaction to hematoxylin, etc., it surrounded itself with (manufactured), or was surrounded by, an increased amount of homogeneous matrix, which remained free from lime salt deposition. The bone corpuscles in contact with the vascular elements, *i.e.* those of the osteoid borders of the trabeculæ, underwent a diminution in size and a change in shape, and tended to revert, if they actually did not do so, to connective tissue cells. At the same time they surrounded themselves with (manufactured), or were surrounded by, an excessive amount of matrix, which remained free from lime salt deposition. In our experience whatever the reaction of the osteoblasts to the abnormal condition of the faulty diet, the same reaction will be manifested by the cartilage cell. The processes of bone resorption so prominent in the skeleton of rats confined for periods of some duration to faulty diets low in calcium seemed to be held in abeyance under the particular abnormal conditions obtaining in these experiments. Only slight evidences of resorptive activity in the calcified portions of the trabeculæ could be found. In the presence of the particular disproportion in the calcium-phosphate ratio characterizing the faulty diets used in these experiments both osteoblast and cartilage cell seemed to undergo changes which made them particularly resistant to the destructive forces ordinarily in operation.

EXPLANATION OF PLATES.

PLATE 4.

FIGS. 1 and 2. Photomicrographs of low magnification of sections from the distal end of the femur of rats on the diet of Lots 3143 and 3133. The photomicrographs show the appearance of bones affected with a very severe rickets. There are broad zones of osteoid tissue around the trabeculæ and the epiphyseal nucleus of ossification and the diaphysis. The cartilage is persistent to a marked degree, forming a wide metaphysis. It contains no calcium salts and is invaded by blood vessels from the shaft. (Rats 679 and 811. Diet of Lots 3143 and 3133.) Objective—Leitz microsummar F-5 full aperture. No ocular.

PLATE 5.

FIG. 3. This picture shows a condition which exactly simulates that found in mild cases of rickets in man. The osteoid borders of the trabeculæ in this bone are much narrower than those of the bones shown in Figs. 1 and 2. The cartilage is persistent and irregularly invaded. There is no zone of provisional calcification. The metaphysis of the bone is narrow and contains small scattered deposits of calcium salts. This picture was taken with the same apparatus used to photograph Figs. 1 and 2. (Rat 642. Diet of Lot 3127)

FIG. 4. To show detail of the growing region of the bone in Fig 3. Note the absence of provisional calcification and the irregular prolongation and invasion of the cartilage. The metaphysis, which is narrow, is made up of trabeculæ of osteoid, and the metaphysis contains irregular deposits of lime salts. Objective—Leitz acromatic No. 3. No ocular.

PLATE 6.

FIG. 5. Photomicrograph with the same apparatus used in making Fig. 4. This picture shows the metaphysis in detail of a bone affected with exaggerated rickets. The very broad metaphysis is composed of osteoid trabeculæ, traversed by a few small marrow spaces, and blood vessels which have sprouted from the vascular tree of the diaphysis. Much of this osteoid tissue was the product of metaplasia of the cells of the epiphyseal cartilage, some of which may be seen embedded in it in a condition of transition into osteoid corpuscles. (Diet of Lot 3133.)

FIG. 6. High power photomicrograph. This picture shows calcified bone, *OS*, surrounded by a zone of osteoid tissue, *OST*. Note the lamination of the osteoid, the small size and wide separation of the osteoid corpuscles and the endothelioid appearance of the osteoblasts which surround the trabeculæ, *O*. (Diet of Lot 3143.) Objective—Leitz acromatic No. 6. No ocular.

PLATE 7.

FIG. 7. This picture shows the cartilage cells in the metaphysis during their transformation into osteoid. In it the fusion of the cells into osteoid tissue in the immediate vicinity of an invading blood vessel is well shown. (Diet of Lot 3133.)

FIG. 8. Section from the distal end of the femur of a rat (No. 819) on the diet of Lot 3137. In this diet 2 per cent of cod liver oil replaced 1.5 per cent of dextrin and 0.5 per cent of butter fat. This bone was in a condition of osteoporosis with essentially normal calcification. There is a provisional calcified zone and no metaphysis has been formed. Only physiological osteoid tissue is present.

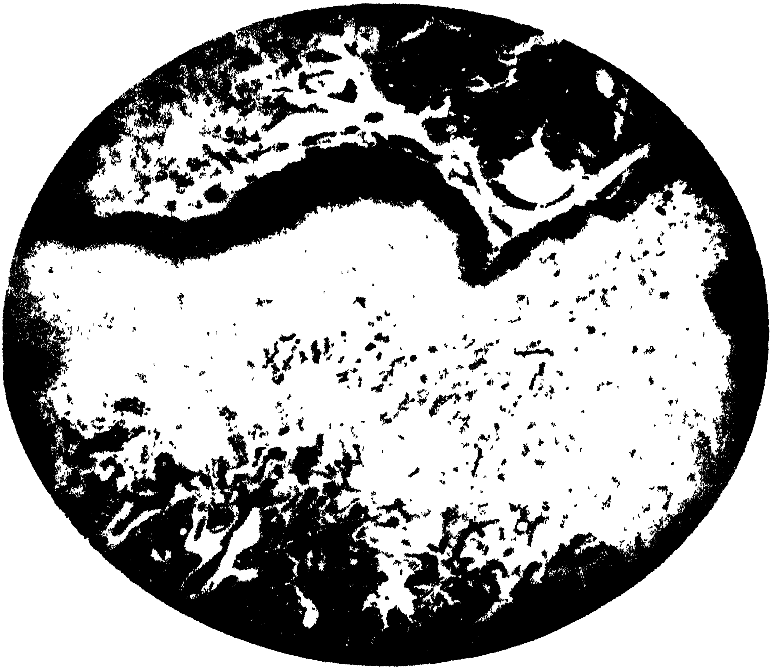
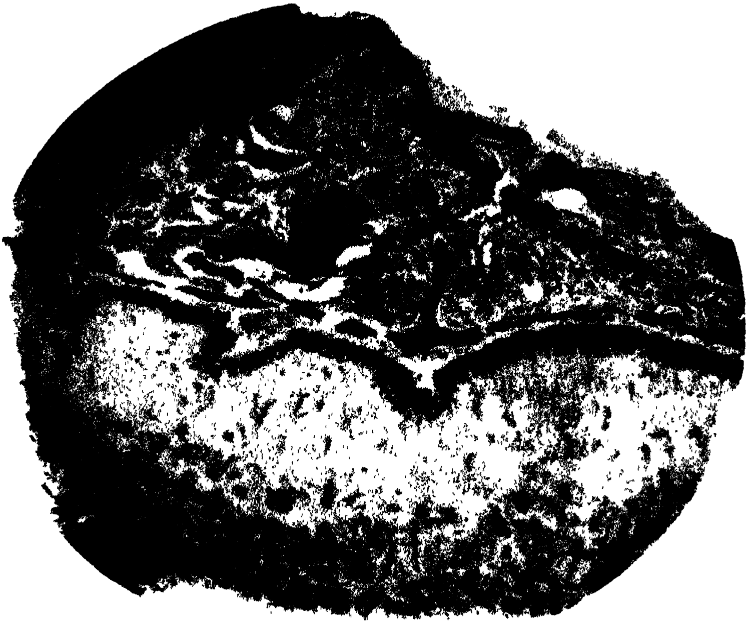


Fig. 1.



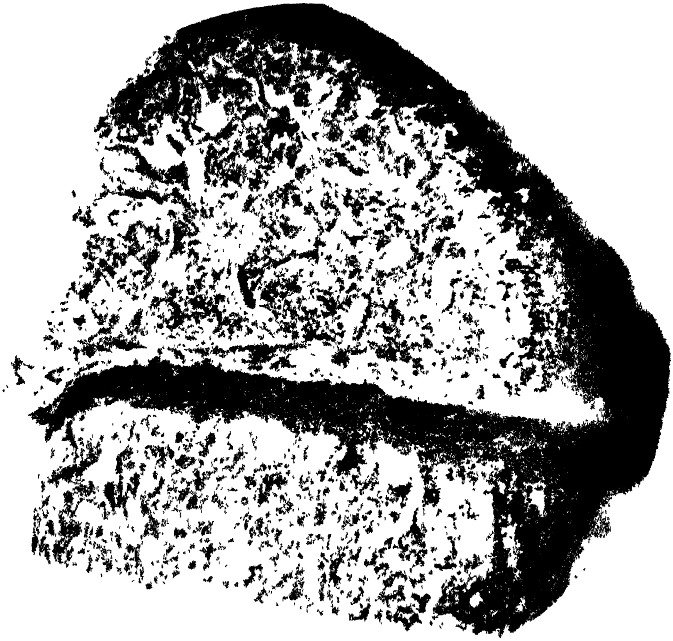


FIG. 3.

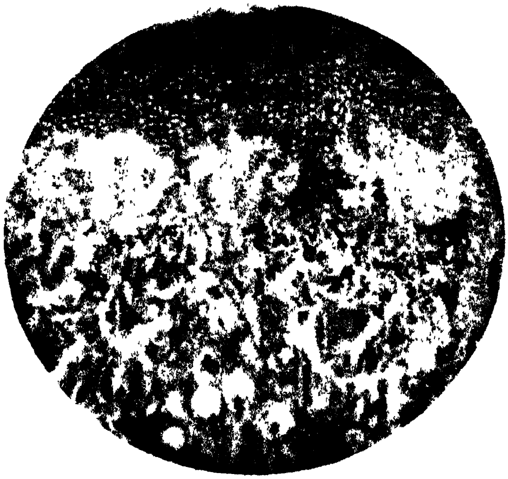


FIG. 4.

(McCollum, Simmonds, Shipley, and Park: Experimental rickets. VIII)

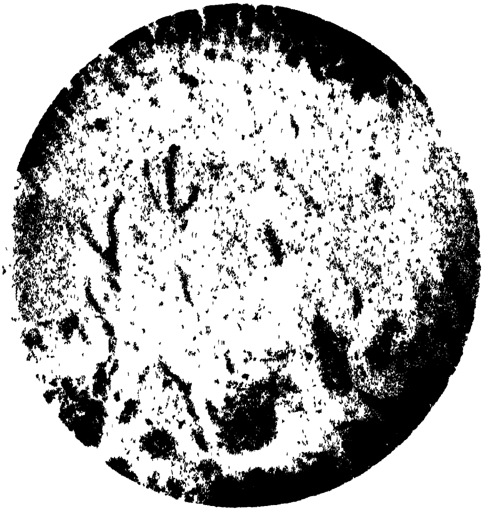


FIG. 5.

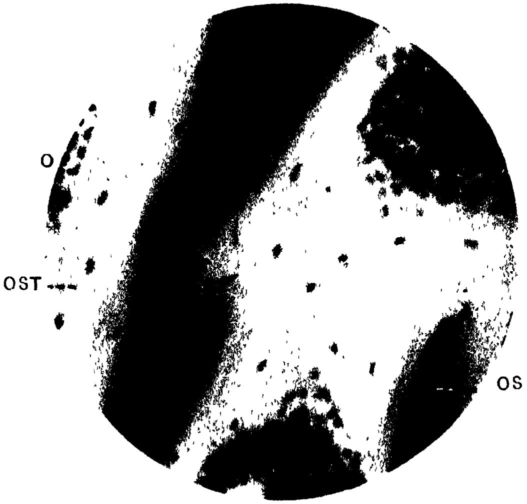


FIG. 6.

(McCollum, Simmonds, Shipley, and Park: Experimental rickets. VIII.)

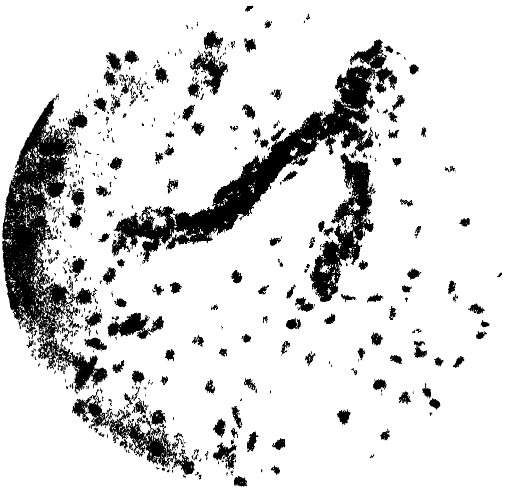


Fig. 7.

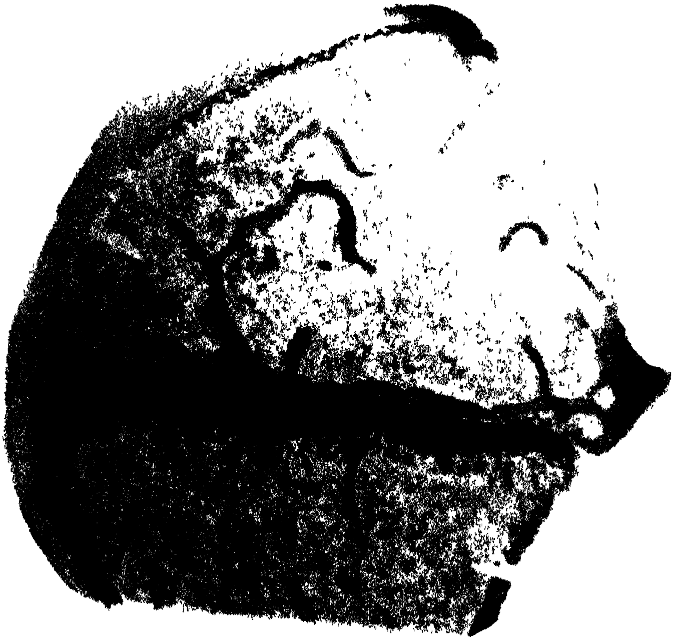


Fig. 8.

(McCollum, Simmonds, Shipley, and Park: Experimental rickets. VIII.)

THE DIFFUSIBLE CALCIUM OF THE BLOOD SERUM.

I. A METHOD FOR ITS DETERMINATION.

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It is now well recognized that the calcium in the plasma does not all exist in the form of simple solution. The first conclusive demonstration of a non-diffusible calcium we owe to Rona and Takahashi (1), who subjected serum to dialysis in which all inorganic salts except the calcium were fully compensated for in the dialyzing fluid outside the membrane, while calcium was present in varying amounts. By such means these authors showed that on the average only 65 per cent of the calcium in the serum was capable of diffusing through a membrane. Very interesting experiments on the state in which calcium occurs in serum were done by Cushny (2), who filtered ox serum through collodion membranes under 150 mm. mercury pressure. He found that, while sodium, potassium, and chlorine filtered as any ordinary solution would, a portion of the calcium was held back. The non-filterable (colloidal) calcium represented 30 to 40 per cent of the total.

This colloidal, non-diffusible calcium is looked upon as a protein combination and while nothing is known regarding its real nature, it seems definite enough to warrant further study. It seemed to us worth knowing whether in conditions of abnormal calcium metabolism, such as rickets or tetany, the amount of the diffusible and non-diffusible calcium might throw any light on the processes involved. Brinkman (3) and Brinkman and Van Dam (4) have recently published observations on the calcium ion concentration in serum and emphasize strongly the importance of its exact maintenance. According to Brinkman, this maintenance of calcium ion concentration depends on the concentration of hydrogen ion and bicarbonate ion. It appeared to us that the amount of the total calcium in solution (diffusible, non-colloidal) might be influenced by differences in $[H^+]$ or $[HCO_3^-]$, since it is perfectly well known that definitely acidifying the serum (as in Lyman's method of calcium determination) results in the total calcium being present in true solution.

In this paper we describe the technique of dialysis together with observations on variation in CO_2 tension under which the dialysis took place, while in the following paper we record the results obtained on the blood in rickets and experimental tetany.

TABLE I.

Showing Dialysis Complete in 24 hours.

Date and name.	Time of dialysis.	CO_2 saturation, PCO_2 mm. Hg.	Ca in serum in 4 cc.		Ca in dialysate in 4 cc		Serum Ca per 100 cc.	Dialyzable Ca.	Remarks.
			Start	End	Start	End.			
	hr.	*mm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
Jan. 20,	24	46.2	0.420	0.384	0.168	0.236	10.5	72	Duplicates
1921.	48	46.2	0.420	0.382	0.168	0.226	10.5	68	
A.M.P.	24	48.3	0.420	0.396	0.209	0.248	10.5	68	"
	48	48.3	0 420	0.394	0.209	0.246	10.5	67	
	24	46.0	0.420	0.414	0.258	0.256	10.5	60	"
	48	46.0	0.420	0.408	0.258	0.266	10.5	65	

In order to carry on our studies, we have devised a method of dialyzing serum against a buffer solution of the Ringer type, at the same time maintaining a constant CO_2 tension in the dialyzing system.

If serum be dialyzed against a calcium-free Ringer buffer solution, there appears to be a progressive dissociation of the colloidal calcium compound, so that calcium continues to diffuse out, until at the end of 7 days 90 per cent has passed into the dialysate. When, however, calcium is added to the dialyzing fluid ("compensation dialysis"), equilibrium is obtained in 24 hours, as Table I shows.

Our method will now be described in detail.

Details of Method.

Collection of Blood for Dialysis.—Blood is obtained by venous puncture into 100 cc. centrifuge tubes, defibrinated by whipping, centrifuged, and the serum pipetted. If not used immediately, the serum is kept stoppered in the ice box without preservative.

Dialyzing Fluid.—Fluid A, used in the early part of the work, was composed of equal volumes of double strength calcium-free Ringer's solution, and M/375 primary and secondary phosphates, having an initial pH of 7.1. The composition of the double strength Ringer's solution was:

NaCl	36.0	gm.
$NaHCO_3$	10.08	"
KCl	1.68	"
Distilled H_2O to	2,000	cc.

The composition of the phosphate solution was:

M/15 KH_2PO_4	15.2	cc.
M/15 Na_2HPO_4	64.8	"

This was diluted 25 times, so that the final concentration of phosphorus was approximately that of inorganic phosphate of blood serum, namely 0.003 gm. per 100 cc.

The hydrogen ion concentration of the Ringer phosphate solution after saturation with 6 per cent CO_2 was found to be pH 7.4. Depression of freezing point = 0.708 (18 per cent hypertonic).

Fluid B was based on analytical figures for normal human plasma obtained from Dr. Greenwald, to whom acknowledgment is hereby made for placing at our disposal his unpublished data, and its composition is given in Table II. Depression of freezing point of this fluid was 0.585 (isotonic). This solution was made up double strength, so that it could be mixed with equal volumes of double strength $CaCl_2$ solutions, as will be described under the method of compensation. The figures in the table represent the value of the single strength solution, ready for use. Calcium figures are not given, for these vary with the compensation desired. It will be noted that the values approximate closely those for normal human plasma, with the exception of the chlorine, which is here in excess of that found normally. The buffer action of this fluid is due mainly to its bicarbonate content. The curve of Fig. 1 illustrates the CO_2 dissociation of the fluid.

TABLE II.

*Composition of Dialyzing Fluid.**

Compound.	Cl	Na	K	Mg	P	Molecular concentration.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
$NaHCO_3$, 252 mg....		69.0				0.03
KH_2PO_4, 8.8 mg.....			2.52		2.0	0.00065
$MgCl_2$, 10.59 mg.....	7.89			2.7		0.0011
KCl, 29.6 mg........	14.1		15.5			0.00397
NaCl, 625 mg..	380.0	246.0				0.1069
Totals..............	401.99	315.0	18.02	2.7	2.0	

*Based on analytical figures for human plasma obtained from Greenwald.

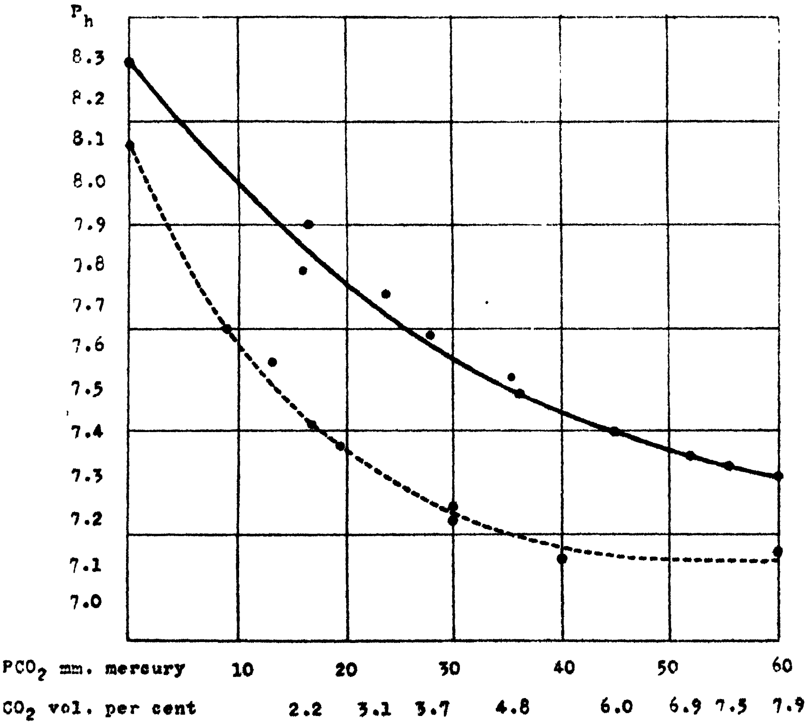

FIG. 1. CO_2 dissociation curve for dialyzing fluid B containing calcium. ——— shows curve of Fluid B. - - - - represents the relation between pH and CO_2 tension in cat's blood, taken from Dale and Evans.

Dialyzing Sacs.—There was considerable difficulty in obtaining satisfactory sacs. Collodion membranes did not hold back protein for a sufficient length of time, and there was a progressive passage of fluid into the sac, presumably an osmotic phenomenon. Later, parchment sacs were obtained. These were Schleicher and Schüll's Diffusions-Hülsen, No. 579A. They proved to be impermeable to protein after 7 days dialysis at ice box temperature, and after 3 days at 48 to 52° C. A series of six sacs was tested for individual variations in permeability, using 0.9 per cent sodium chloride against distilled water. No significant differences in rate of diffusion of chlorine ion were found. Nor was there passage of fluid into the sac containing serum as determined by inserting a graduated capillary tube into the fluid within the sac. Before using, the sacs were soaked over night in 5 per cent hydrochloric acid and washed free of acid with distilled water, and allowed to dry in an open flask covered with filter paper. The same procedure was followed with the used sacs, so as to remove all possible traces of calcium in the sac wall.

Pipettes.—To obtain greater accuracy, special pipettes to conform to the requirements of the Bureau of Standards, were made for us by E. Leitz, Inc. These pipettes, measuring 4 cc. to the tip, and graduated in $\frac{1}{10}$ cc. are straight with inside diameter of 5 mm. They were used for measuring the serum and dialyzing fluid before and after dialysis.

Dialyzing Tubes.—Special Pyrex glass tubes were made for this work, $3\frac{1}{2} \times 1$ inches. The parchment sacs were suspended in these by means of cotton threads, threaded through the upper rim of the sac. The threads were looped around glass rings encircling the upper end of the sac, so as to prevent the sac from leaning against the tube, and so allowing the dialyzing fluid to creep up the sac wall.

Regulation of CO_2 Tension.—Before dialysis, both serum and dialyzing fluid were saturated with CO_2 of known tension, and this tension was maintained throughout the duration of the dialysis. CO_2-air mixtures of any desired proportion were obtained by driving measured volumes of air and CO_2 through separate gas meters fitted with precision gauges, into a 20 liter rubber bag. After allowing time for diffusion, the CO_2 content of the mixture was checked with a Fredericia apparatus. The composition as determined by analysis usually varied from the theoretical by less than 0.2 volume per cent. Where the error was greater than this, the bag was emptied and refilled. For analysis, a volume of approximately 1,500 cc. was passed through the apparatus.

Saturation of the dialysate and of the serum was performed by exposing 5 cc. of fluid in a 500 cc. separatory funnel through which 1,500 cc. of the gas mixture were passed (over moist glass beads). The fluid was shaken for 2 minutes. Care was taken in transferring the fluid to the dialyzing tubes to avoid unnecessary exposure to the air.

Method of Compensation.—A calcium chloride solution was made up roughly to contain the same calcium concentration as serum. The calcium was quantitatively determined by Lyman's method (5) and checked by the titration method. A quantity of the solution representing the desired amount of calcium was pipetted into the dialyzing tube and evaporated to dryness, first on the water bath and then in the oven, and cooled in the desiccator. The calcium-free dialyzing fluid was then added to the dried calcium chloride. This way of adding the calcium seemed of great advantage, in that any desired amount of the calcium chloride could be pipetted and dried. It was at once found, however, that a large error had crept in and that the analytical figures for the calcium in the dialyzing system were too high. The following experiment was then performed. 3 cc. of the calcium chloride solution (= 0.273 mg. of calcium) were pipetted into the Pyrex dialyzing tube and dried as described. The dry residue was then dissolved in 3 cc. of the dialyzing fluid which had been saturated with 6 per cent CO_2-air mixture and gave a pH of 7.4. The clear solution was then analyzed for calcium. Duplicates so treated and analyzed gave these results.

Tube A, from which 2.5 cc. of solution were taken for analysis, showed 0.248 mg. of calcium; the amount of calcium in the original calcium chloride solution was 0.228 mg.

Tube B. In 2.6 cc. of the redissolved calcium chloride, 0.260 mg. of calcium was found; while 2.6 cc. of the original solution contained 0.237 mg.

This experiment showed us the source of our error. Calcium, even in so small amount as 0.02 mg. (Tube A) and 0.023 mg.

(Tube B) was dissolved from the glass during the drying process. So small an amount would hardly seem to vitiate any results, but when working with only 2 cc. of serum, which contains about 0.2 mg. of calcium, it introduces an error of 10 per cent.

To avoid this error, the calcium was added by mixing with the dialyzing fluid B an equal volume of calcium chloride standard solution. These solutions were made up in four different concentrations:

Solution A, to contain 40 per cent of the serum calcium, using 10.5 mg. per 100 cc. as an average figure. Analysis, 2 cc., 0.168 mg.

Solution B, to contain 50 per cent of the serum calcium. Analysis, 2 cc., 0.210 mg.

Solution C, to contain 60 per cent of the serum calcium. Analysis, 2 cc., 0.258 mg.

Solution D, to contain 70 per cent of the serum calcium. Analysis, 2 cc., 0.297 mg.

When these calcium chloride solutions (double strength) were added in equal volume to the double strength dialyzing fluid B, there was a slow precipitation of the calcium carbonate, and so the solutions were kept separate until immediately before being used. Saturation with the CO_2-air mixture prevented this precipitation.

Technique of Dialysis. 4 cc. of saturated dialyzing fluid are pipetted from the separatory funnel into the dialyzing tube, which has been previously filled with the same CO_2 mixture as that used for saturation. It is then corked. The pH of the dialysate is determined with the excess of dialyzing fluid in the funnel by the colorimetric method of Levy, Rowntree, and Marriott (6). 4 cc. of the saturated serum are pipetted into a *dry* sac which is then suspended by the attached threads in the dialyzing fluid, but with the serum level above that of the outer fluid level. This is done because when the sac has become thoroughly moistened, it expands and the level inside falls, while that outside rises. The tube is then closed with a paraffined cork, through which passes a glass tube. The air in the tube is replaced by the CO_2-air mixture, an outlet being provided by a slit in the cork, and the tube sealed with paraffin. The duplicate tube having been set up in the same way, the two are placed in a tightly stoppered museum jar, which is also filled with air-gas mixture, and sealed with paraffin. Fig. 2 shows the apparatus set up.

Calcium Determinations.—As much of the serum and dialysate as it is possible to recover are pipetted (usually 3.5 to 3.6 cc.), the pH of the dialysate determined as before dialysis, and the calcium in each is determined. The clear dialysate was treated in exactly the same manner as the dialyzed serum. In every case, where protein had leaked through, the material was discarded.

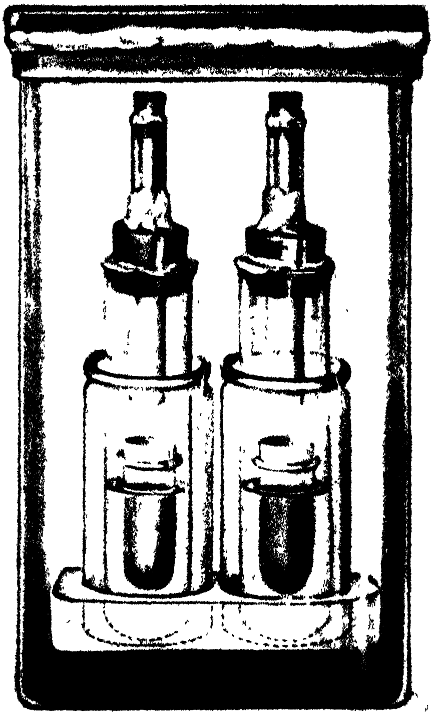

FIG. 2. Apparatus used in determination of diffusible serum calcium.

Calculation of Results.—For this, the amount of calcium dialyzed, the calcium added to dialysate before dialysis, and the undialyzed serum calcium must be known. This is expressed in the formula

$$\text{Diffusible Ca} = \frac{(\text{Ca in dialysate after dialysis} \times 2) - \text{Ca added to dialysate}}{\text{Original serum Ca in amount used}}$$

Tables III to V show the results obtained. It will be seen in Table III that in a range of CO_2 saturation of serum from 17 mm. of mercury tension to 62 mm. there is no alteration in the percentage of diffusible calcium of the serum.

Table III also shows that a change in the hydrogen ion concentration of dialysate from pH 7.6 before dialysis to pH 7.0 after dialysis exerts no influence on the percentage of diffusible calcium.

TABLE III.

Varying CO_2 Tensions.

Date and name.	CO_2 saturation, PCO_2 mm. Hg.	pH of dialysate.		Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Dialyzable Ca.	Total serum Ca per 100 cc.	Remarks.
		Before	After	Start.	End.	Start	End.			
	mm.			*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*mg*	
Feb. 2, 1921.	28.0	7.6	7.33	0.444	0.420	0.258	0.278	67	11.1	Dialyzing fluid was hypertonic.
A.M.P.	46.2	7 4	7.1	0.420	0.408	0.258	0.266	65	10.5	Fluid A.
	60.6	7.3	7.0	0.444	0.410	0.258	0.292	73	11.1	
Feb. 21, 1921.	17.6	7.6	7.5	0.440	0.448	0.295	0.296	67	11.0	Isotonic dialyzing fluid.
E.E.W.	43.5	7.45	7.35	0.440	0.424	0.295	0.306	72	11.0	
	62.0	7.2	7.0	0.440		0.295	0.300	70	11.0	Fluid B.

SUMMARY.

A method is described for determining diffusible serum calcium. Values are given for normal human and normal dog serum.

CONCLUSIONS.

1. The diffusible calcium of the serum of normal men and dogs was found to comprise from 60 to 70 per cent of the total serum calcium.
2. Varying the CO_2 saturation of the serum between 17 mm. mercury tension and 62 mm. does not alter this percentage.

TABLE IV.

Dialyzable Calcium of Normal Bloods.

Date and name	CO_2 saturation.	pH of dialysate.		Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Dialyzable Ca.	Serum Ca per 100 cc.	Remarks.
		Before.	After	Start.	End	Start	End			
1921	*mm. Hg*			*mg*	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	
Jan. 20,	46.2	7.4	7.1	0.420	0.408	0.258	0.266	65	10.5	Fluid A.
A.M.P.	28 0	7.6	7.33	0.440	0.420	0.258	0.278	67	11.1	
Feb. 8, E.C.K.	46.3	7.4	7.3	0.421	0.398	0.258	0.272	67	10.5	Fluid B.
Feb. 17, H.J.W.	44.8	7 37	7.3	0.412	0.424	0.295	0.288	68	10.3	
Feb. 21, E.E.W.	43.5	7.45	7.35	0.440	0 424	0.295	0 306	72	11.0	
Mar. 12, Mrs. S.	45.5	7.43	7.25	0.416	0.420	0 295	0 300	73	10.4	
Feb. 28, Dog 1.	45.2	7.4	7.15	0.438	0.424	0.295	0.296	68	10.9	Considerable hemolysis.
Mar. 3, Dog 2.	43 7	7 4	7.0	0.444	0.434	0.295	0 302	69	11.1	Considerable hemolysis.
Mar. 7, Dog 3.	45.0	7.3	7.2	0.430	0.440	0.295	0 276	60	10.7	Slight hemolysis.
Mar. 21, Dog 4.	45.0	7.4	7.15	0.424	0.436	0.295	0.276	61	10.6	No hemolysis.
Mar. 24, Dog 5.	43.7	7.4	7.15	0.408	0.384	0.254	0.268	69	10.2	" "

TABLE V.

Duplicate Analyses, Experimental Tetany, Dog 6.

Series NN.	Ca in serum in 4 cc.		Ca in dialysate in 4 cc.		Serum Ca in 100 cc.	Total Ca in system in 8 cc.			Dialysable Ca.
	Start.	End.	Start.	End.		Before	After.	Difference.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	0.248	0.274	0.210	0.184	6.1	0.458	0.458	0.0	63
2	0.248	0.274	0.210	0.180	6.1	0.458	0.454	0.004	60
3	0.248	0.218	0.105	0.128	6.1	0.353	0.346	0.007	61
4	0.248	0.232	0.105	0.124	6.1	0.353	0.356	0.003	58

BIBLIOGRAPHY.

1. Rona, P., and Takahashi, D., *Biochem. Z.*, 1911, xxxi, 336.
2. Cushny, A. R., *J. Physiol.*, 1919–20, liii, 391.
3. Brinkman, R., *Biochem. Z.*, 1919, xcv, 101.
4. Brinkman, R., and Van Dam, E., *Koninklijke Akad. v. Wetensch. t. Amst., Proceedings*, 1919, xxii.
5. Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.
6. Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.

THE DIFFUSIBLE CALCIUM OF THE BLOOD SERUM.

II. HUMAN RICKETS AND EXPERIMENTAL DOG TETANY.

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In the preceding communication (1), there was reported in detail the technique employed in this work. It is our purpose in this paper to record the results of the study of the blood calcium in rickets and experimental tetany.

Rickets.

In the consideration of the pathology of rachitic bones and cartilage, there would seem to be four possibilities to account for the failure of proper ossification. First, that there is a deficiency of calcium in the diet or a failure of absorption of a sufficient supply; second, that there is an alteration of the form of the blood calcium, making it less available for the normal formation of bone; third, that there are absent intermediary products, possibly of the ductless glands, possibly other salts, particularly phosphates, which are necessary for the deposition of calcium in the bones; and fourth, that there is an alteration, chemical or otherwise, in the osteoid tissue and provisional cartilage cells or matrix, such that calcium, even though it may be supplied in sufficient and proper form by the blood, cannot be taken up by these cells. Or there may be a combination of any or all of these possibilities. It is to the second that we have directed particular attention.

Since the work of Howland and Marriott (2) in 1917, it has been known that there is a slight diminution of the serum calcium in rickets. They found in twenty-one cases an average of 9.4 mg. of Ca per 100 cc. of serum, whereas the normal is always above 10 mg. Their lowest figure is 7.9 and in five other cases the Ca was less than 9 mg. We also have found a definite reduction of the serum calcium in rickets. Thus, five cases showed: 9.8, 8.7, 8.5, 9.0, and 7.6 mg. of Ca per 100 cc. of serum, respectively. The electrical reactions of these cases were normal, except in the first two, which showed anodal hyperexcitability.

The determination of the diffusible calcium in rachitic blood was carried out in precisely the same manner as that for normal blood described in our first paper.

Attention is directed to Table I, which gives the analytical figures. Both cases presented clinically, as well as by x-rays of the long bones, evidences of active rickets.

TABLE I.

CO_2 saturation P-CO_2.	pH of dialysate.		Ca in 4 cc of dialysate.		Ca in 4 cc of serum.		Serum Ca per 100 cc.	Total Ca in system			Dialyzable Ca.
	Before.	After	Before.	After	Before	After		Before.	After	Difference	
Baby L. F. Age 11 months.											
			mg	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg*	*mg.*	*per cent*
45.5	7.35		0.295	0.272	0.362	0.386	9.0	0.657	0.658	+0.001	68
45.5	7.4		0.295	0.268	0.362		9.0	0.657			66
45.5	7.4		0.210	0.210	0.362		9.0	0.572			58
Baby R. M. Age 12 months.											
44.6	7.4	7.4	0.295	0.244	0.302	0.334	7.6*	0.597	0.578	−0.019	63
45.0	7.35	7.3	0.295	0.246	0.302		7.6				65

* Electrical reactions normal.

Tetany.

It is hardly necessary to state that the blood calcium in infantile tetany and experimental tetany has been found markedly reduced. MacCallum and Voegtlin (3), MacCallum and Vogel (4), Howland and Marriott (2), Binger (5), and others are all agreed in this respect.

MacCallum, Lambert, and Vogel (6) in 1914 made the following statement: "If tetany blood be dialyzed under exactly the same conditions as normal blood, it still loses a proportionate amount of the calcium, which would perhaps show that it is not especially the loss of diffusible calcium as contrasted with a non-diffusible form which is important in producing tetany."

We have sought in view of the increased CO_2-combining power of the plasma in tetany, reported by some investigators, to correlate the hypothesis discussed in the previous paper (1) with the low calcium content of tetany blood.

For this purpose, dogs were used, removing parathyroids, and in all but one case the thyroids as well.

The CO_2-combining power of the plasma in the tetany dogs was determined. Dog 4 had a preoperative combining power of 57.9 volumes per cent. During active tetany, it had fallen to 41.5 volumes per cent. Dog 6 showed practically no change. Dog 7 before operation showed 58 volumes per cent. 2 days later, without tetany, the combining power had risen to 69 volumes per cent. Later during a convulsion, it fell to 39 volumes per cent. In the presence of this definite acidosis, the diffusible calcium was still 66 per cent, so that it is apparent that Brinkman's hypothesis does not apply to the diffusible calcium.

Dog 4.—Female. Mar. 21. Blood was drawn under oil from jugular vein. The CO_2-combining power of the oxalated plasma, saturated with alveolar air, was 57.9 volumes per cent (Van Slyke micro method). Blood was also taken for determination of dialyzable calcium. This was not citrated, but was defibrinated by whipping (Table II).

Mar. 26. Under ether anesthesia, complete thyroparathyroidectomy was performed.

Mar. 28. Dog showed twitching of various groups of muscles, and the reflexes were hyperactive. Respirations were deep and rapid. Blood drawn at 10.30 a.m., before anesthesia, showed a plasma CO_2-combining power of 41.5 volumes per cent. Dog was then etherized, and blood drawn and defibrinated as before for determination of dialyzable calcium (Table II).

Dog 6.—Male. Apr. 4. Blood plasma CO_2-combining power was 57 volumes per cent, when saturated with 5.5 per cent CO_2-air mixture. The serum calcium was 10.2 mg. per 100 cc.

The dog was then etherized and complete thyroparathyroidectomy done.

Apr. 6. Early in the morning the dog was found in convulsions, and he had been vomiting. Respiration and pulse rates were very rapid. Slight twitchings of the abdominal muscles could be felt, but reflexes were not obtainable. CO_2-combining power was 54.1 volumes per cent when saturated with a 6.0 per cent CO_2-air mixture.

Blood was taken for determination of dialyzable calcium, Table III.

TABLE II.

Dog 4.

CO_2 saturation P-CO_2.	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialysable Ca.
	Before.	After.	Before.	After	Before.	After.		Before	After.	Difference.	
Before operation.											
			mg.	*mg.*	*mg*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
45	7.4	7.15	0.295	0.276	0.424	0.436	10.6	0.710	0.712	+0.002	61
45	7.4	7.15	0.295	0.274	0.424	Lost.	10.6	0.710			59
45	7.37	7.25	0.258	0.252	0.424	0.440	10.6	0.682	0.692	+0.010	58
2 days after operation. Thyroparathyroidectomy.											
45	7.4	7.25	0.210	0.184	0.254	0.282	6.35	0.464	0.466	+0.002	62
45	7.42	7.2	0.210	0.180	0.254	0.282	6.35	0.464	0.462	−0.002	59
45	7.4	7.25	0.105	0.136	0.254	0.230	6.35	0.359	0.366	+0.007	65
45	7.4	7.2	0.105	0.114	0.254	0.244	6.35	0.359	0 358	−0.001	48*

* This is an exceptional figure, possibly due to variation in the sac used.

TABLE III.

Dog 6.

2 days after operation. Thyroparathyroidectomy.

CO saturation P-CO_2	pH of dialysate.		Ca in 4 cc. of dialysate		Ca in 4 cc. of serum		Serum Ca per 100 cc.	Total Ca in system.			Dialyzable Ca
	Before.	After.	Before.	After	Before.	After.		Before.	After	Difference.	
			mg.	*mg*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
45.7	7.4	7.15	0.210	0.184	0.248	0.274	6.1	0.458	0.458	0.0	63
45.7	7.38	7.2	0.210	0.180	0.248	0.274	6.1	0.458	0.454	−0.004	60
45.7	7.4	7.2	0.105	0.128	0.248	0.218	6.1	0.353	0.346	−0.007	61
45.7	7.42	7.2	0.105	0.124	0.248	0.232	6.1	0.353	0.356	+0.003	58

TABLE IV.

Dog. 7.

4 days after operation. Thyroparathyroidectomy.

CO_2 saturation P-CO_2.	pH of dialysate.		Ca in 4 cc. of dialysate.		Ca in 4 cc. of serum.		Serum Ca per 100 cc.	Total Ca in system.			Dialysable Ca.
	Before.	After.	Before.	After.	Before.	After.		Before.	After.	Difference.	
			mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
45	7.43	7.3	0.210	0.194	0.270	0.288	6.7	0.480	0.482	+0.002	66
45	7.42	7.25	0.210	0.196	0.270	0.286	6.7	0.480	0.482	+0.002	67
45	7.42	7.2	0.180	0.186	0.270	0.268	6.7	0.450	0.454	+0.004	71

Dog. 7.—Male. Apr. 9. CO_2-combining power of plasma saturated with 6 per cent CO_2-air mixture was 58 volumes per cent, and the serum calcium 10.3 mg. per 100 cc. Under ether anesthesia, the right thyroid, both upper parathyroids, and right lower parathyroid were removed.

Apr. 11. Dog showed slightly hyperactive knee jerks, and a CO_2-combining power of 69.2 volumes per cent (saturated with 6 per cent mixture).

Apr. 12. Left thyroid and remaining parathyroid were removed.

Apr. 13. At 9 a.m. the dog had a general convulsion. CO_2-combining power of plasma, saturated with a 6 per cent CO_2-air mixture was 39 volumes per cent. Dog was etherized and blood was drawn from the jugular vein by cannula and defibrinated for calcium determination, Table IV.

Dog. 8.—Male. May 2. Plasma CO_2-combining power was 45 volumes per cent, and the serum calcium was 10.7 mg. per 100 cc. The dog was etherized and right thyroid, both right parathyroids, and left lower parathyroid were removed. The parathyroids were calcified.

TABLE V.

Dog 8.

14 days after operation. Partial thyroparathyroidectomy.

CO_2 saturation P-CO_2.	pH of dialysate		Ca in 4 cc of dialysate.		Ca in 4 cc of serum.		Serum Ca per 100 cc	Total Ca in system.			Dialyzable Ca.
	Before	After	Before	After.	Before.	After.		Before	After	Difference.	
			mg	*mg*	*mg.*	*mg*	*mg*	*mg.*	*mg.*	*mg*	*per cent*
44.7	7.4	7.25	0 210	0.214	0 336	0.336	8.4	0.546	0.550	+0.004	65
44 7	7.4	7.3	0.210	0 218	0.336	0.346	8.4	0 546	0.564	+0.018	67
45.5	7.42	7.3	0.127	0.182	0.336		8.4				71
45 5	7.4	7.35	0.127	0.182	0.336		8.4				71

May 10. The dog showed slight twitching of abdominal muscles and spasticity of legs.

May 11. Serum calcium was 7.2 mg. per 100 cc.

May 12. Condition noted on May 10 has persisted, but there have been no convulsions, nor active tetany.

The dog was etherized and blood taken for calcium determination as in previous experiments, Table V.

SUMMARY.

In two cases of rickets, with serum calcium of 9.0 and 7.6 mg. per 100 cc., the percentage of diffusible calcium was found to be between 58 and 70 per cent, within the range found in normal subjects.

In four cases of experimental tetany in dogs, similar percentages of dialyzable calcium were found, 58 to 71 per cent, with serum calciums of 6.1 to 8.4 mg. per 100 cc. Two of these dogs showed a reduced CO_2-combining power of the plasma at the time the calcium determinations were made, showing that this form of acidosis does not affect the diffusible calcium. It, therefore, appears that, in so far as can be determined by *in vitro* experiment, the reduced serum calcium in experimental tetany is not due to a lowering of the diffusible as contrasted with the non-diffusible form. The proportion between the two remains constant in the presence of a reduced total. Also, this proportion does not change with varying CO_2-combining powers of the plasma.

CONCLUSIONS.

The diffusible calcium of the serum in experimental tetany in the dog and human rickets is between 60 and 70 per cent of the total serum calcium.

BIBLIOGRAPHY.

1. von Meysenbug, L., Pappenheimer, A. M., Zucker, T. F., and Murray, M. L., *J. Biol. Chem.*, 1921, xlvii, 529.
2. Howland, J., and Marriott, W. McK., *Quart. J. Med.*, 1917-18, xi, 289.
3. MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.
4. MacCallum, W. G., and Vogel. K. M., *J. Exp. Med.*, 1913, xviii, 618.
5. Binger, C., *J. Pharmacol. and Exp. Therap.*, 1917, x, 105.
6. MacCallum. W. G., Lambert. R. A., and Vogel, K. M., *J. Exp. Med.*, 1914, xx. 149.

THE OXYGEN DISSOCIATION OF HEMOGLOBIN, AND THE EFFECT OF ELECTROLYTES UPON IT.

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Barcroft (1) has gathered extensive data on the dissociation of oxyhemoglobin. Plotting oxygen tension against percentage saturation with oxygen, he has drawn dissociation curves, and, by developing the aggregation theory in conjunction with Hill (2), he has sought to explain divergences in their form. Incidentally they have represented mathematically the curves for the dissociation $HbO_2 \rightleftharpoons Hb + O_2$ by a general equation $K = \frac{[Hb] \cdot [O_2]^n}{[HbO_2]}$ and have determined constants which fit the equation in the case of every experimental curve.

The data of Barcroft and Camis (3) have shown that in whole blood divergences in form of the curves are due to variations in the salt content of the blood from various species and individuals; but that, on the other hand, preparations of crystallized hemoglobin made at different times give sensibly similar curves. Barcroft and Roberts (4) have demonstrated that dialyzed hemoglobin gives curves which differ from those for crystallized hemoglobin, but which are identical for dialyzed preparations from different animals. These curves they have regarded as representative of pure hemoglobin. Barcroft and Means (5) have shown that carbonic acid shifts the dissociation curve of dialyzed hemoglobin and have measured the shift quantitatively.

Barcroft (1) and L. J. Henderson (6) have suggested that hemoglobin in alkaline solution, or alkali hemoglobinate, has a smaller dissociation constant for oxygen than has acid hemoglobin. And Henderson has by an indirect method calculated the ratio of two constants for the following equations in the case of whole blood:

$$HHbO_2 \rightleftharpoons HHb + O_2 \qquad K_A = \frac{[HHb]\cdot[O_2]}{[HHbO_2]}$$

$$BHbO_2 \rightleftharpoons BHb + O_2 \qquad K_S = \frac{[BHb]\cdot[O_2]}{[BHbO_2]}$$

showing that K_A is greater than K_S. Henderson concludes: ". . . . oxyhemoglobin must be a stronger acid than reduced hemoglobin. Of course it also follows that the salts of hemoglobin must have a greater affinity for oxygen than has acid hemoglobin itself."

To obtain direct experimental evidence upon this point, the effect of electrolytes and non-electrolytes on the dissociation curve of oxyhemoglobin was studied by us at the suggestion of Professor L. J. Henderson.

Methods.

Fresh defibrinated beef blood was centrifuged, and the corpuscles thrice washed with 0.9 per cent sodium chloride solution. The corpuscular magma, placed in freshly made collodion sacs of about 100 cc. capacity, was dialyzed against running tap water for 48 hours. Dialysis was continued on ice for one or more days against several changes of distilled water. The sacs were usually closed securely to diminish dilution of the contents.

In order to rid the hemoglobin of metallic cations one of the following procedures, suggested by Professor Henderson, was usually adopted. Either (*a*) the solution was poured from the sacs and saturated with carbon dioxide, dialysis being continued against distilled water; or (*b*) the sacs were placed intermittently in distilled water saturated with carbon dioxide. In both cases the hemoglobin was finally dialyzed against pure distilled water. It was supposed that this treatment with carbonic acid would free hemoglobin from the cations at its isoelectric point, according to the equation $BHb + H_2CO_3 \rightleftharpoons BHCO_3 + HHb$.

The freedom of the dialyzed solutions from electrolytes was roughly estimated by measuring conductivity at 25°C. The lowest conductivity reached, 8×10^{-5} reciprocal ohms, is the lowest conductivity reported in the literature when it is considered that the concentration of the hemoglobin was 16 per cent. Rarely did the conductivity exceed 3×10^{-4} reciprocal ohms, which is 4 per cent of that of whole blood, or equivalent to that of a 0.0027 M potassium chloride solution.

The hemoglobin solutions contained, on the average, 14 per cent hemoglobin, as measured by the oxygen combined with the hemoglobin after saturating it with pure oxygen at atmospheric pressure, and assuming a molecular weight of 16,700. Except for a diminution in conductivity, no difference could be detected in the behavior of solutions which had been treated with carbon dioxide during dialysis as compared with those not treated.

The solutions of hemoglobin were equilibrated with mixtures of carbon dioxide-free hydrogen and of air, selected to give about 50 per cent saturation of the hemoglobin with oxygen. This was done in a rubber-stoppered bottle of 1 liter capacity, provided with two outlet tubes. Through the shorter, a sample of solution could be withdrawn without exposure to the outside air; the other was provided for gas sampling. 20 to 25 cc. of hemoglobin solution, evacuated by boiling with a water pump at 35 to 40°C., were run in from a pipette to the bottle, which had been previously filled with the gas mixture. Electrolytes and non-electrolytes, in solutions of suitable concentration were introduced through the rubber stopper from a graduated Luer syringe. The equilibrator bottle was then rotated continuously for 15 to 30 minutes in a thermostat at 38°C. The rotation was interrupted to adjust the pressure to that of the atmosphere. The same solution was often equilibrated five times.

After equilibration the bottle was inverted in order to collect the solution in its neck, thereby presenting only a small surface to the gas above it. A gas sample of 10 cc. was then taken into an analyzer of the type described by Y. Henderson (7), and analyzed for carbon dioxide and oxygen. Some of the stock gas mixture was then admitted into the bottle at atmospheric pressure and room temperature, and a 2 cc. sample of solution run into a Mohr pipette. The oxygen content was determined by the blood-gas method of Van Slyke (8). A second sample of solution was saturated with pure oxygen at atmospheric pressure and room temperature in a separatory funnel, and the oxygen capacity was determined. This was determined once or twice in a series of successive equilibrations. Van Slyke's method is not well suited to measuring the percentage saturation of hemoglobin with oxygen, since two samples must be taken for each determination. The results of many workers seem to show that the quantities of oxygen found, though consistent among themselves, are too high. It was, however, the most available method. Great care was taken to correct calculations upon each analysis for dissolved gases, and to determine the errors due to manipulation and reagents.

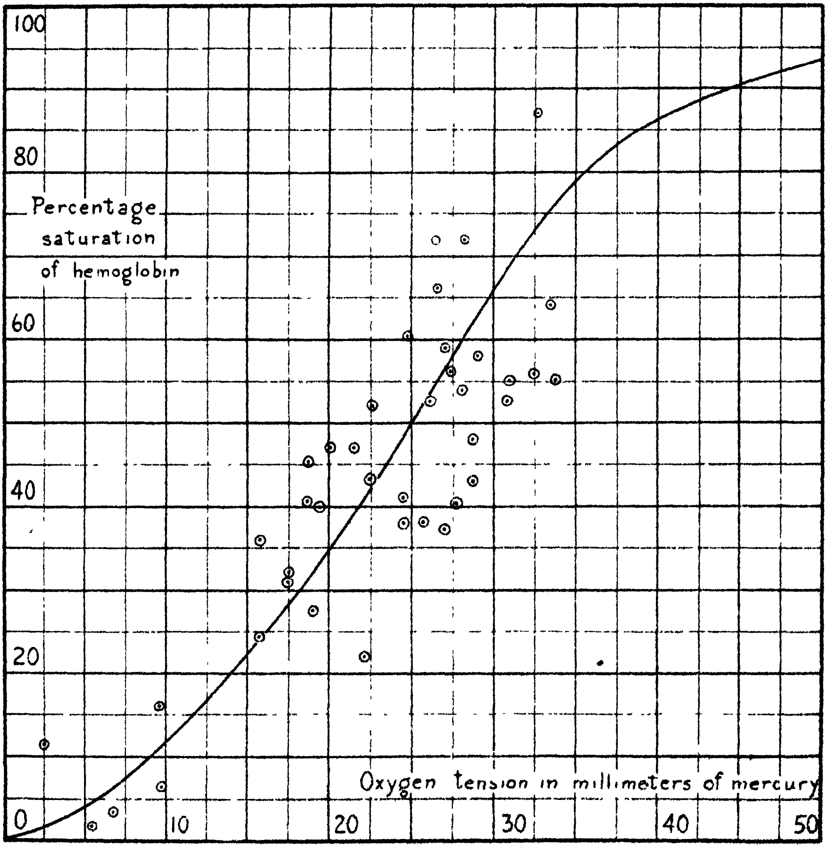

Fig. 1.

EXPERIMENTAL RESULTS.

Dialyzed Hemoglobin.—Some 40 determinations of percentage saturations were made upon 26 different solutions prepared from the blood of 4 animals. These are plotted against oxygen tension in Fig. 1, and an arbitrary curve has been drawn through these points. It is a reflexed or S-shaped curve, and resembles Bohr's (9) dissociation curve for crystallized hemoglobin.

Blood samples from the same animal prepared simultaneously gave solutions exhibiting the most uniform results. There is, however, no close agreement among the other points, nor was any to be expected from the varying conditions. Most of the points to the right of the line were obtained in the latter part of the investigation, and it is probable that these points are to be explained by the fact that the gas analyses occasionally revealed small tensions of carbon dioxide, 1 to 8 mm., which were reckoned in with the oxygen.

The individual points bear no demonstrable relation to the purity of the hemoglobin as measured by conductivity, nor to the use of carbon dioxide during dialysis. The variations among corresponding points may be due in part to differences in hydrogen ion concentration, to very slight differences in the electrolyte content, and to bacterial action. None of these factors was adequately controlled.

Addition of Acid.—Lactic acid and carbonic acid (Fig. 2, B and A) lowered the amount of oxygen combined at a given oxygen tension (30 mm.), and the effect was increased by increasing concentrations of acid. This change was reversible, and could be nearly restored by the addition of an equivalent amount of alkali. Concentration of lactic acid greater than 0.015 M caused a gradual diminution of oxygen capacity due to an irreversible change in the hemoglobin.

Barcroft and Means (5) have demonstrated this effect of carbonic acid in varying concentrations upon dialyzed hemoglobin. Barcroft and Orbeli (10) have shown that lactic acid lowers the amount of oxygen taken up by whole blood.

Addition of Alkali.—Solutions of sodium hydroxide (Fig. 2, B) from 0.001 to 0.04 M caused more oxygen to be taken up by the hemoglobin at a given tension. This change was reversible upon the addition of an equivalent amount of acid. It increased with the amount of alkali added. Similar results were obtained with disodium phosphate, Na_2HPO_4, from 0.01 to 0.1 M (Fig. 2, D). These results confirm those of Barcroft and Camis (3) with ammonia, disodium phosphate, and sodium bicarbonate added

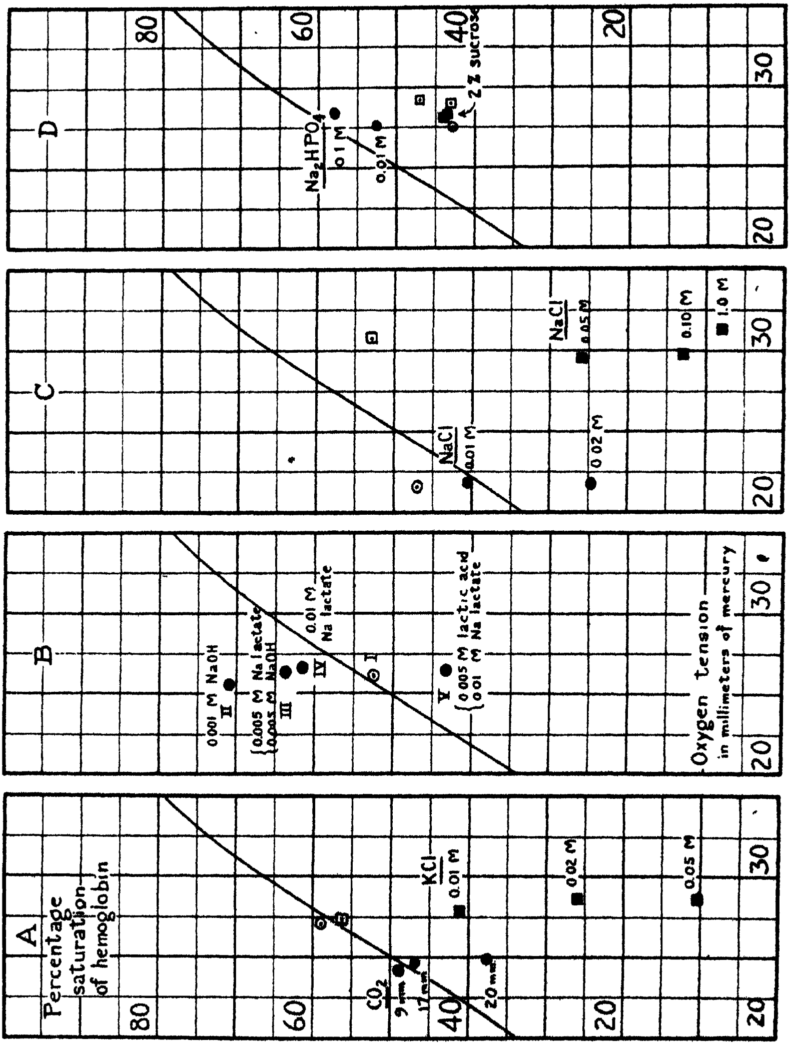
A
B
C
D
Percentage saturation of hemoglobin
80
60
40
20
20
30
Oxygen tension in millimeters of mercury
CO_2
9 mm
17 mm
20 mm
KCl
0.01 M
0.02 M
0.05 M
0.001 M NaOH
II
0.005 M Na lactate
0.005 M NaOH
III
0.01 M Na lactate
IV
I
V
0.005 M lactic acid
0.01 M Na lactate
NaCl
0.01 M
0.02 M
NaCl
0.05 M
0.10 M
1.0 M
Na_2HPO_4
0.1 M
0.01 M
2% sucrose

Fig. 2.

to crystallized hemoglobin; and those of Momose (11) with sodium hydroxide and ammonia added to whole blood.

Addition of Neutral Salts.—Potassium and sodium chlorides at concentrations from 0.01 to 1.0 M reduced the oxygen bound at a given oxygen tension (Fig. 2, A and C). This effect increased with increasing concentration, at first rapidly and then more gradually. A solution containing both sodium and potassium chlorides had the same effect as a solution of either alone.

Addition of Non-Electrolytes.—Neither urea from 0.1 to 1.0 M nor sucrose of 2 per cent (Fig. 2, D) affected the oxygen dissociation of the dialyzed hemoglobin solutions. These results confirm those for urea of Poulton and Ryffel (12) and of Momose (11) both of whom used whole blood.

SUMMARY.

Our results apparently differ from those of Barcroft in two respects. Our dialyzed solutions of hemoglobin give S-shaped curves comparable to those of Bohr (9) for crystallized hemoglobin. Barcroft's hyperbolic curves for dialyzed hemoglobin were obtained when ammonium carbonate or hydroxide was added,[1] and we have obtained comparable points when other alkalies were added. Barcroft has recently[1] obtained curves similar to ours for dialyzed hemoglobin to which no alkali was added.

Secondly, Barcroft (3) found that the addition of neutral salts to crystallized hemoglobin increased the amount of oxygen taken up by it at a given tension of oxygen. We have found the reverse to be the case with our samples of dialyzed hemoglobin. Very recently Barcroft[1] has made the same observation. Our results were obtained only at oxygen tensions of about 30 mm., and we are unable to predict the result at higher tensions.

DISCUSSION.

The chemical behavior of hemoglobin has been the subject of much speculation. The data of Barcroft (1); Douglas, Haldane, and Haldane (13); Roaf (14); and others, have been clearly interpreted on the basis of Hill's (2) aggregation theory as applied to the mass action principle. The implications of the aggregation theory, which we believe serves as a foundation for understanding the chemistry of hemoglobin, have been ably set forth (1, 2, 13).

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[1] We are greatly indebted to Mr. Barcroft for communicating the results of his recent experiments to one of us.

Up to the present time little has been said of the dependence of the equilibrium between oxygen and hemoglobin upon that between hemoglobin and electrolytes as such. The present experiments suggest that the degree of electrolytic dissociation of hemoglobin, as of other proteins (15), is variable, and that it is markedly different for alkali hemoglobinates and for acid hemoglobin. The effect of acid in diminishing the amount of oxygen or carbon monoxide bound by hemoglobin at a given tension, the effect of alkali in increasing the saturation with oxygen at a given tension, and the fact that oxyhemoglobin behaves as if it were a more highly dissociated acid than reduced hemoglobin (6, 16, 17) give support to this view.

Certain facts are explicable upon both hypotheses, such as: the influence of neutral salts, the absence of effects due to non-electrolytes, and the increased osmotic pressure of hemoglobin in alkaline solution.

It seems probable that the electrolytic dissociation of hemoglobin must be considered as supplementary to its aggregation in giving to hemoglobin its remarkable range of variation in physicochemical activity. The conception of hemoglobin as an ionized substance is the only view so far suggested to account for the effects of acid and of alkali upon hemoglobin.

Unfortunately the present data are inadequate for more than a qualitative discussion. It is hoped that work now in progress will give a quantitative definition of the effects due to ionic equilibria.

We are indebted to Professor L. J. Henderson for much valuable aid and criticism.

CONCLUSIONS.

1. A technique is described for dialyzing hemoglobin, depending upon its protein property of acting as a dissociable electrolyte.
2. Hemoglobin prepared by several procedures for dialysis gives S-shaped curves for oxygen dissociation.
3. Addition of alkali with formation of alkali hemoglobinate results in an increase of the oxygen bound by hemoglobin at a constant tension.

4. Neutral salts even in small concentrations decrease the amount of oxygen bound.

5. Non-electrolytes have no effect upon the oxygen equilibrium.

6. The equilibrium between oxygen and hemoglobin is a function of that between hemoglobin and electrolytes.

BIBLIOGRAPHY.

1. Barcroft, J., The respiratory function of the blood, Cambridge, 1914.
2. Hill, A. V., *J. Physiol.*, 1910, xl, p. iv.
3. Barcroft, J., and Camis, M., *J. Physiol.*, 1909–10, xxxix, 118.
4. Barcroft, J., and Roberts, F., *J. Physiol.*, 1909–10, xxxix, 143.
5. Barcroft, J., and Means, J. H., *J. Physiol.*, 1913–14, xlvii, p. xxvii.
6. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.
7. Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 31.
8 Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
9. Bohr, C , *Centr. Physiol.*, 1903, xvii, 682.
10. Barcroft, J., and Orbeli, L., *J. Physiol.*, 1910–11, xli, 355.
11. Momose, G , *Biochem. J.*, 1915, ix, 485.
12. Poulton, E. P., and Ryffel, J. H., *J. Physiol.*, 1913, xlvi, p. xlvii.
13. Douglas, C. G., Haldane, J. S., and Haldane, J. B. S., *J. Physiol.*, 1912, xliv, 275.
14. Roaf, H. E., *J. Physiol.*, 1909, xxxviii, p. i.
15. Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.
16. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
17. Parsons, T. R., *J. Physiol.*, 1917, li, 440.

ANIMAL CALORIMETRY.

SEVENTEENTH PAPER.

THE INFLUENCE OF COLLOIDAL IRON ON THE BASAL METABOLISM.

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(Received for publication, June 1, 1921.)

INTRODUCTION.

The biological action of inorganic hydrosols has been extensively studied during the years which have elapsed since Credé[1] introduced the use of colloidal silver into medicine. From these investigations it seems very probable that the action of the inorganic hydrosols is much the same as the action of small doses of salts of the same metals (see the literature by Bechhold[2]).

The colloidal metal dissociates slowly in water into metal ions, and the bactericidal action, the property which has been the most studied, is supposed to be due to these dissociated ions. After intravenous injection the colloids are distributed all over the body within ½ to 1 hour and, while still in the colloidal state, are deposited in almost every organ with subsequent slow dissociation of ions.

Investigations on the influence of these substances upon the metabolism are very rare. It has been found that silver hydrosol increases the protein metabolism.[3]

A special interest is connected with the negative iron oxide hydrosol. While the positive iron oxide hydrosol, according to

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[1] Credé, B., *Arch. klin. Chir.*, 1897, lv, 861.

[2] Bechhold, H., Die Kolloide in Biologie und Medizin, Dresden and Leipsic, 2nd edition, 1919.

[3] Bechhold,[2] p. 402.

Bechhold, unites with the negative blood colloids, forming an irreversible gel, the negative iron oxide hydrosol easily mixes with serum without producing any coagulation. This colloid solution is said to act as an oxygen carrier and to have properties similar to hemoglobin.[4]

The purpose of this investigation has been to examine the influence of such an intravenously injected colloidal iron solution on the basal metabolism.

Methods.

Two dogs, trained for calorimeter work, were used in the experiments. The dogs were fed once daily with the "standard diet."[5]

The bladder was emptied and washed before and after each experiment.

The hydrosol was injected into the jugular vein, and immediately thereafter the dog was placed in the calorimeter. In each experiment 5 cc.[6] were used.

The movements of the dog in the calorimeter were recorded on a smoked drum.

The efficiency of the calorimeter was controlled by frequent alcohol checks.

Basal Metabolism.

The standard diet was administered at 5 p. m. the day before the experiment. Thus, the basal metabolism was determined 18 to 20 hours after feeding.

In all experiments the dog was perfectly quiet, and the temperature of the calorimeter was between 25 and 26°C.

The basal metabolism of Dog 20 was found to average 14.92 calories per hour and the respiratory quotient 0.80 (Table I).

The basal metabolism of Dog 18 was found to be 16.18 calories per hour and the respiratory quotient 0.80.

The Influence of Colloidal Iron Intravenously.

The results of intravenous injection of 5 cc. of colloidal iron are given in Table II. The average total heat production per hour of Dog 20 was 16.00 calories and the average respiratory quotient 0.84. One of the experiments, No. 10-A, was performed immediately after the basal metabolism was determined, on the same day.

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[4] Bechhold,[2] p. 417.

[5] Lusk, G., *J. Biol. Chem.*, 1912–13, xiii, 185.

[6] Made by Laboratoires Clin, Paris.

After administering colloidal iron the heat production of Dog 18 was found to be 18.72 calories per hour and the respiratory quotient 0.82.

TABLE I.

Basal Metabolism of Dog 20.

Date.	Experiment No.	Indirect calorimetry	R Q
1921			
Apr. 7	3	14.45	0.81
May 6	5	14.77	0.79
" 9	7	14.94	0 81
" 13	10	15.25	0.78
" 14	11	15.22	0.81
Average		14.92	0 80

TABLE II.

Colloidal Iron Intravenously—Dog 20.

Date	Experiment No.	Indirect calorimetry.	R Q.
1921			
Apr. 8	4	15.63	0.85
May 7	6	15.79	0.87
" 13	10-A	16.40	0.81
Average		16.00	0 84

The total heat production was thus increased about 7 per cent in Dog 20 and about 15 per cent in Dog 18.

The average CO_2 output per hour of Dog 20 after the injection was 5.51 gm., but only 4.89 gm. in the basal metabolism. The average O_2 consumption was 4.68 gm. per hour after injection and 4.46 gm. in the basal metabolism.

In Dog 18 the CO_2 production was increased from 5.37 to 6.35 gm. per hour and the O_2 consumption from 4.87 to 5.63 gm. per hour after the injection.

TABLE III.

Dog 20.

Standard diet given at 5 p.m. the previous day. The results are expressed in average values per hour.

Date.	Experiment No	Time	CO_2	O_2	R Q	H_2O	Urine N.	Calories			Remarks
								Protein.	Non-protein	Total heat calculated	
1921			*gm*	*gm*		*gm*	*gm*				
Apr. 7	3 Basal.	11.22–2.22	4.80	4.33	0.81	11.06	0.082	2.18	12.27	14.45	Alcohol check Apr. 4, average R. Q. 0.665. Weight 8.47 kg. Dog quiet.
" 8	4 Colloidal iron intravenously.	11.32–2.32	5.52	4.65	0.85	12.86	0.095	2.52	13.11	15.63	Weight 8.47 kg. Colloidal iron intravenously, 5 cc. Dog quiet.
May 6	5 Basal.	11.18–2.18	4.77	4.40	0.79	12.15	0.098	2.62	12.15	14.77	Alcohol check May 5, average R. Q. 0.667. Weight 9.20 kg. Dog quiet.
" 7	6 Colloidal iron intravenously.	11.57–1.57	5.58	4.47	0.87	11.47	0.105	2.80	12.99	15.79	Weight 9.10 kg. Colloidal iron intravenously, 5 cc. Dog quiet.
" 9	7 Basal.	12.02–2.02	4.92	4.39	0.81	11.12	0.098	2.60	12.34	14.94	Weight 9.10 kg. Dog quiet.

May 13	10 Basal.	11.10– 1.10	4.91	4.60	0.78	15.43	0.071	1.90	13.35	15.25	Alcohol check May 10, average R. Q. 0.668. Weight 9.30 kg. Dog quiet.
" 13	10-A Colloidal iron intravenously.	2.40– 5.40	5.43	4.92	0.81	12.96	0.077	2.03	14.38	16.40	5 cc. colloidal iron intravenously 20 min. after the last experiment. Between these experiments the dog was catheterized, but received no food or drink. Dog quiet.
" 14	11 Basal.	10.25–12.25	5.04	4.57	0.81	11.32	0.079	2.09	13.13	15.22	Weight 9.20 kg. Dog quiet.

TABLE IV.

Dog 18.

Standard diet given at 5 p.m. the previous day. The results are expressed in average values per hour.

Date.	Experiment No.	Time.	CO_2	O_2	R. Q	H_2O	Urine N	Calories.			Remarks.
								Protein.	Non-protein	Total heat calculated	
1921			*gm.*	*gm.*		*gm.*	*gm*				
May 17	85 Basal.	12.25– 2.25	5.37	4.87	0.80	7.86	0.107	2.85	13.33	16.18	Weight 9.45 kg. Dog quiet.
" 18	86 Colloidal iron intravenously.	11.36– 1.36	6.35	5.63	0.82	12.37	0.137	3.65	15.07	18.72	Colloidal iron intravenously, 5 cc. Weight 9.45 kg. Dog quiet.

The respiratory quotient was slightly higher in both dogs during the injection experiments than during the determinations of the basal metabolism.

There was no marked increase of the protein metabolism. The chief increase of the total heat production fell on the non-protein metabolism.

The results are summarized in Tables III and IV.

CONCLUSIONS.

1. Intravenous injections of iron hydrosol in dogs cause an increase of the O_2 consumption and the CO_2 production. The average increase of the heat production in one of these dogs was 7 per cent and in one experiment on the other the increase was 15 per cent.
2. The increased metabolism coincides with a slight increase of the respiratory quotient.
3. The chief increase of the total heat production falls on the non-protein metabolism.

I wish to express my sincerest thanks to Professor Graham Lusk for advice and help in running the calorimeter, and to Mr. James Evenden for technical assistance.

CHEMICAL FACTORS IN FATIGUE.

I. THE EFFECT OF MUSCULAR EXERCISE UPON CERTAIN COMMON BLOOD CONSTITUENTS.*

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The beginning of the truly chemical study of muscular exercise and fatigue may be traced back to Du Bois Raymond who, in 1859, showed that contracting muscles become acid as a result of their activity. Others, following closely, pointed out that not only is lactic acid formed in the working muscle but there is a simultaneous disappearance of carbohydrates, notably glycogen, the partial oxidation of which presumably gives rise to the acid.

Space does not permit of an extended review of the work which followed from then till the present day, but a few of the outstanding features may be noted.

Ranke, in 1865, first investigated the effect upon the contractility of surviving, excised muscles produced by perfusion with solutions of various substances. His experiments were later extended and elaborated by Lee, Burridge, and others with the result that a number of substances were found whose influence upon surviving muscle produced to a certain extent the recognized phenomena of fatigue, as evidenced by a decrease in power of contraction. There have been reported from time to time among these so called "fatigue products" (some or all of which may possibly be formed in muscles during their activity and produce the condition of fatigue) the following: lactic and oxybutyric acids and their alkali salts, HCl, H_2SO_4, CO_2, NaH_2PO_4, KH_2PO_4, phenol, indole, skatole, and potassium ions.

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*A thesis presented to the Department of Chemistry of Leland Stanford Junior University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Weichardt, in 1904, claimed to have isolated a specific fatigue toxin, similar to the bacterial toxins, which produced all the symptoms of fatigue when injected into the blood stream. He purported furthermore to have isolated an antitoxin, by regular immunological methods, but his results have never been confirmed.

The excretion of substances in the urine during and following muscular work has received considerable attention.

It seems to be generally conceded that no increase in nitrogen metabolism accompanies muscular work. Nevertheless, a number of workers have reported substantial increases in nitrogen elimination for some time following a period of strenuous activity. The current opinion of the independence of nitrogen metabolism is therefore not without question. The commonly reported increases in sulfur and phosphorus elimination may also have a bearing on this point.

The question of changes in the excretion of nitrogenous extractives is still unsettled. Creatine and creatinine are apparently not involved in any regular way, but uric acid and the purines have in general been found to increase after work.

The acidity of the urine is generally, but not invariably, found to increase, as might be expected from the formation of lactic acid in the muscles and the lowering of the alkaline reserve of the blood.

The output of carbon dioxide is invariably increased in exercise, which is important when the demonstrated fatigue-producing action of carbon dioxide on the muscles is borne in mind.

Sugar is practically the only constituent of the blood which has been quantitatively followed during muscular exercise, but there is no agreement between the results from different sources.

A number of secondary physiological concomitants of fatigue have been reported, such as an increased number of red and of white corpuscles.

In summarizing the whole question we may lay down certain general principles: (1) Substances giving rise to hydrogen ions, such as lactic and oxybutyric acids, monopotassium phosphate, and carbon dioxide, are causal agents in fatigue. (2) Certain products of protein disintegration, indole, skatole, and phenol, are apparently capable of producing fatigue symptoms and may be closely related to normal fatigue. (3) There is some evidence that certain negative ions, as the lactate and oxybutyrate, as well as certain positive ions, especially potassium, may be causal agents in fatigue. (4) There is no evidence substantiating Weichardt's theory, still largely quoted, of specific fatigue toxins. (5) The formation and excretion of a number of materials such as uric acid, purines, urea, etc., may be influenced by muscular work and fatigue, without their standing in any causal relation to fatigue.

EXPERIMENTAL.

The present study was undertaken with the purpose of determining, by means of improved biochemical methods of reasonable accuracy, the changes which occur in the more common constituents of the blood during fairly severe muscular exercise. The investigation will later be extended to other substances, and other conditions.

A certain amount of preliminary work was done with dogs as subjects, but the results were so irregular and the conditions of the experiments so difficult to control that it was entirely abandoned in favor of human subjects, nearly all of whom were university students. A few sets of results were obtained with running as a mode of exercise. These data, as far as they go, are given in Table I. Later it was deemed advisable to lengthen the period of exercise and two subjects undertook a 65 mile bicycle ride, extending over about 12 hours and including a considerable amount of hill-climbing. The data covering this period are given in Table II. A consideration of the results from these widely differing types of activity, especially as regards the changes in blood sugar, suggested the possibility that the human organism differs in its reaction to short strenuous effort and to long tedious work. Accordingly it was decided to divide the muscular work into two kinds, a short, strenuous period of activity, and an easier, but longer period. For the first type rapid stair-climbing was chosen—a mode of exercise often made use of in this connection. A double flight of stairs in the laboratory building, about 20 feet in height, was used, and the subjects ran up and down these stairs as rapidly as possible until nearly exhausted. The total time of the exercise did not exceed 15 minutes in any case. For the other type of exercise bicycle riding was chosen. Two subjects generally rode simultaneously and at such speed that they were in a distinct state of fatigue at the end of from 2 to 3 hours. Most of the subjects were not accustomed to riding long distances.

TABLE I.*

Subject	Total nitrogen.			Non-protein nitrogen per 100 cc			Urea nitrogen per 100 cc.			Sugar per 100 cc.			Uric acid per 100 cc.		
	S	R	K	S	R	K	S	R	K	S	R	K	S†	R	K
	per cent	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*mg*	*mg.*	*mg*	*mg*	*mg*	*mg*		*mg*	*mg*
I	3.37	3.55	3.35	49.0	39.6	45.3	19.2	14.8	20.7	118	86	110	<	2.3	
II	3.49	3.56	3.44	(45.6)	40.6	47.1	19.7	13.6	18.7	97	86	108	=	3.7	
III	3.57	3.62	3.40	49.4	44.9	46.7	20.0	15.0	18.9	127	131	131	>	5.1	
IV		3.72	3.51		46.4	50.0		18.6	23.2		92	111		7.6	

* Of the horizontal rows numbered with Roman numerals I contains the results from the samples taken before the exercise; II, after the subject had run 100 yards; III, after running one mile; and IV, after 2½ hours subsequent rest. The vertical columns contain the results belonging to each of the three subjects, S, R, and K.

† The results in this column are only qualitative. The samples were not compared in the colorimeter, but II showed distinctly more uric acid than I, and III distinctly more than II.

TABLE II.*

Subject.	Time.	Total N.	Non-protein N per 100 cc.	Urea N per 100 cc.	Uric acid per 100 cc.	Sugar per 100 cc.
		per cent	*mg.*	*mg.*	*mg*	*mg*
S	Before.	3.33	39.7	17.5	4.5	115
	After.	3.63	50.2	25.1	12.5	79
R	Before.	3.40	39.6	22.0	2(?)	106
	After.	3.48	43.9	29.3	3.7	100

* Showing results before and after a 65 mile bicycle ride, occupying 12 hours. A small amount of food was taken during that time, principally four or five pieces of bread and butter, but the second sample was taken 5 hours after eating.

It was decided to confine the attention to the changes in sugar, uric acid, non-protein and urea nitrogen, preformed and total creatinine, and cholesterol. These were determined in the plasma as well as in the whole blood, whence an insight was obtained into the changes in the distribution of these materials between plasma and corpuscles. Total nitrogen was determined in the preliminary experiments, but this was not extended into the major portion of the work. In addition to these chemical determinations it was considered important to note the changes in certain physical properties of the blood, especially such as would throw light upon any possible variations in the blood volume. A direct determination of the changes in blood volume was originally undertaken with dogs, but was necessarily given up when the work was carried over to human subjects.

The experiments were generally carried out in the late afternoon, not closer than 3 to 4 hours from the noon meal. For collecting the blood samples a paraffined 50 cc. flask was used, fitted with a two-hole stopper carrying two right-angled glass tubes about 2 inches in length. One of these was connected with a piece of heavy rubber tubing through which the operator could exert suction at will; the other tube was paraffined internally and connected by a short piece of rubber tubing to a 20 gauge needle. A small amount of powdered potassium oxalate was introduced into the flask and 40 to 50 cc. of blood were drawn from an arm vein. A preliminary, or control, sample was taken in this way and another one, similarly, after the exercise.

Methods.

Chemical Determinations.—For the determination of sugar, uric acid, non-protein nitrogen, urea, and creatinine, both preformed and total, the general procedure of Folin and Wu (1) was adopted because of its simplicity, reasonable accuracy, and economy cf material. The proteins were precipitated both in plasma and whole blood in exactly the same manner, and 15 cc. of whole blood and 10 cc. of plasma yielded ample protein-free filtrate in each case to make determinations of sugar in duplicate or triplicate, urea and non-protein nitrogen in triplicate, total creatinine in duplicate, and preformed creatinine and uric acid in single determinations. Because of the uncertainty in preparing and keeping active urease solutions and because of the large number of samples urea was determined by the autoclave modification, *i.e.* autoclaving at 150°C. for 10 minutes, in which case the total creatinine determinations could be started at the same time.

One more or less important departure from the original Folin-Wu method was made in the case of non-protein nitrogen. Largely as a matter of personal preference, but also because of some difficulty with cloudy solutions and refractory precipitates after digestion with the phosphoric-sulfuric mixture, non-protein nitrogen was determined as follows:

To 5 cc. of the protein-free filtrate in a large Pyrex test-tube about 0.5 gm. of potassium sulfate was added, one or two small pieces of broken porcelain, and 1 cc. of a 1:1 solution of sulfuric acid containing 1 gm. of copper sulfate per 100 cc. The solution was then evaporated to fumes of H_2SO_4, the tube covered with a 2 cm. watch-glass, and digested slowly for 5 minutes after the solution had become clear and bluish. It was then cooled and diluted to about 25 cc. After the addition of 5 cc. of 20 per cent NaOH, a drop of paraffin oil, and a little finely granulated zinc, the ammonia was distilled over into 5 cc. of twentieth normal HCl in a test-tube graduated at 50 cc. and Nesslerized as usual with 5 cc. of the reagent as prepared by Folin and Wu. For the distillation, the test-tube was fitted with a two-hole rubber stopper carrying in one hole an outlet tube to a 6 inch, three-bulb condenser, and in the other hole a 5 cc. pipette closed with a short piece of rubber tubing and a pinch-cock, which served as a means of introducing the NaOH after the test-tube was in position. The solution in the tube was gradually brought to boiling with a microburner and the air slowly expelled from the apparatus. The distillation was carried on for 4 minutes after the condenser began to deliver. Toward the end the apparatus was allowed to cool slightly and the acid to suck back for a short distance. The pinch-cock, closing the pipette in the distillation tube, was then opened for a moment to draw back into the tube any traces of ammonia which might have been trapped within the pipette. Distillation was then continued for a few moments longer before stopping and washing.

Although this method is somewhat longer than the direct Nesslerization, it is easily possible to distil ten to twelve samples in 1½ hours. With ordinary care there is no loss during distillation; the method recovers ammonia quantitatively and is highly satisfactory. A blank determination of the reagents is of course necessary.

The same distilling apparatus above described was used for urea. For receiving the distillate the Folin blood sugar tubes are excellently adapted, being graduated at 25 cc. and having a constriction in the neck at the proper place to prevent any loss of ammonia by too rapid bubbling during the early stages of the distillation. Trouble with excessive frothing is generally traceable to the quality of paraffin oil used, and finely granulated zinc is preferable to quartz or porcelain to prevent bumping.

Cholesterol was determined by the method of Myers and Wardell (2), this procedure being found most satisfactory after trying out several of the colorimetric methods now in use. The only important modification made was the use of a standard solution of pure cholesterol in chloroform for the colorimetric comparison, in place of naphthol green B recommended by the authors of the method.

Specific Gravity.—The Hammerschlag method was first tried but was abandoned in favor of the Westphal balance, since ample material was available. For this purpose a round glass plummet was made having a volume of about 2 cc. This was suspended on a silk thread from the beam of the analytical balance, and by determining its weight in air, water, and blood, the specific gravity was calculated in the usual manner.

Viscosity.—A capillary pipette was made which delivered its contents of blood in 40 to 60 seconds. A fairly rapid delivery was advisable to avoid the settling of the corpuscles. This pipette was carefully calibrated with water and its time-of-delivery curve determined over a 10° range of temperature. Conditions did not permit of a very accurate temperature control of the blood, but it was found sufficiently accurate to use the average of the blood temperatures before and after the determination. It was ascertained that a change of 2 or 3° made practically no difference in the delivery time for blood, and the temperature regulation was well within these limits. For the measurement of the plasma viscosity the ordinary type of capillary viscosimeter was used, immersed in a constant temperature bath. The delivery time for plasma was about 2 minutes. All the data for viscosity are relative to water.

Hemoglobin.—The method of Cohen and Smith (3) was modified by pipetting 1 cc. of blood into about 200 cc. of water in a 250 cc. graduated flask. Then 25 cc. of normal HCl were added and the contents made up to the mark with water. Since only the relative, and not the absolute, amounts of hemoglobin were of interest two samples of blood were treated in this way, one taken before and one after the exercise, and the resulting colors of the acid hematin simply compared against each other in the colorimeter to obtain the "hemoglobin ratio," which is calculated on the assumption that the blood had a content of 100 per cent before the exercise.

Relative Corpuscle Volume.—The relative volume of corpuscles was determined in the customary way by the hematocrit method. The suggestions of Sundstroem and Bloor (4) in regard to the construction of hematocrit tubes, were more or less closely followed. A number of capillary tubes were cut about 11 cm. in length and ground flat at one end with emery. These were all filled from the same sample of blood, centrifuged for the same time, and those which indicated concordant corpuscle volumes accepted for use. A more rigid method of checking the regularity of bore was used in some cases, as follows: A short column of mercury was drawn up into a tube and the length of this mercury column carefully measured as it was moved to different positions within the capillary. If little or no variation in the length of the mercury column was observed the bore of the tube was considered sufficiently regular. When it was possible the same capillary was used for the second blood sample as for the first and centrifuged in each case for the same time at the same speed (1 hour at 2,000 R.P.M.). In other cases two capillaries, calibrated or checked against each other, were used to contain blood from the two samples, respectively, and centrifuged simultaneously for 1 hour at 2,000 revolutions per minute, at the end of which time the length of column was constant. Therefore, the comparative nature of these results is practically certain.

Plasma-Corpuscle Relations.—The concentrations of the various substances in the corpuscles were calculated in the usual manner from the plasma and whole blood concentrations. It must be kept in mind that the determination of corpuscle concentrations by difference in this way leaves considerable opportunity for the accumulation of errors in the corpuscle results, in which there is consequently less certainty than in the directly determined values for plasma.

DISCUSSION.

A consideration of the data in Table I, which are more or less fragmentary, points out certain preliminary facts.

Total nitrogen exhibited a slight tendency to rise during short exercise, which apparently continued for some time after. Non-protein and urea nitrogen underwent no regular change excepting a possible slight increase after the exercise was over. These changes are in accordance with the heretofore observed tendency for the nitrogen elimination to increase *after* work.

Sugar was increased considerably by an amount of exercise represented by a mile run but returned to normal within 2½ hours. Uric acid was noticeably increased by short exercise and continued to increase for some time after. This also agrees with the general finding in regard to uric acid eliminated in the urine.

Table II, representing a much longer work period, presents a somewhat different picture. Total nitrogen and non-protein nitrogen, as well as urea, were increased. The concentration of uric acid rose considerably, but the longer exercise caused a drop in the blood sugar.

The more exhaustive investigation, represented in Tables III and IV, substantiates these preliminary conclusions and goes somewhat further. In order to facilitate comparison of the effects of the two kinds of exercise the average results in the two cases are brought together in Table V.

Let us consider each factor separately.

Sugar.

The shorter exercise period, which represented an expenditure, on the average, of about 58,000 foot pounds of energy in 10 minutes, resulted in an increase in whole blood sugar in seventeen cases out of eighteen. This increase averaged 0.036 per cent. The one instance of decrease was in the case of a subject who exhibited at another time an increase of about the same amount. It may possibly be significant that this subject, a boxer in training, was in the best physical condition of any who participated.

TABLE III.*

Short Period of Exercise.

No.	Subject.	Sugar.			Uric acid			Urea nitrogen.			Non-protein nitrogen.		
		Whole blood.	Plasma	Corpuscles	Whole blood.	Plasma.	Corpuscles.	Whole blood	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles
1	♂NWR	110 178	96 178	129 178							43.3 43.1	38.1 37.4	50.7 50.6
1′	♂NWR	86 143	75 142	100 144	3.5 4.3			15.0 16.7	16.7 19.2	12.9 13.6	35.0 35.1	27.6 31.7	44.3 39.1
1″	♂NWR	104 127	97 121	113 134	3.2 3.6	4.2 5.0	1.9 1.9						
2	♀ACA	94 162	98 160	88 165	6.6 6.5								
2′	♀ACA	100 129	92 121	112 140	2.9 2.6	2.9 4.6	2.9 0	14.4 14.7	14.2 14.6	14.7 14.8	29.6 30.5	24.0 24.2	38.0 39.1
3	♀BBR	87 125	89 126	85 124	4.5 5.4			16.9 16.6			36.0 37.2		
4	♂KDG	95 131	86 124	96 138	5.9 5.9			27.9 28.2	29.0 28.7	26.6 27.7	49.4 48.8	46.0 46.6	53.5 51.0
5	♀MEC	90 144			3.0 3.1	3.7 4.0	2.0 1.5	32.1 25.2			39.2 35.3		

6	♂ELG	94	145	81	136	113	151	5.0	5.7					21.8	21.8	21.9	20.7	21.7	23.1	41.7	41.8	32.5	31.0	55.0	55.3
7	♂FAC	106	167	110	168	101	166	3.6	4.0	3.75	5.1	3.4	2.8	17.2	16.8	16.5	17.3	18.0	16.2	33.4	32.2				
8	♀MEG	79	132					3.5	3.8					21.3	21.3										
9	♂WGB	98	112	89	110	112	115	5.6	6.0	5.75	6.3	5.4	5.6	24.5	22.9	25.8	26.4	22.9	18.3	47.9	48.9	37.4	37.9	61.1	63.5
9′	♂WGB	114	102	114	93	114	113			5.0	5.9			28.5	28.4	31.7	32.0	24.3	23.9						
10	♀EEA							2.6	2.8	1.4	1.65	4.5	4.5	13.9	13.8	14.7	15.5	12.7	11.3						
10′	♀EEA	94	118	87	107	104	131	3.1	3.2	3.5	4.5	2.5	1.6							27.1	27.8	19.4	19.6	38.0	37.6
11	♂ABS	78	88	80	84	76	92			4.4	6.3			16.5	16.1	17.0	17.2	16.0	15.2	43.0	42.4				
12	♂MWG	97	132	98	130	96	137	2.9	3.3	2.6	5.3	3.3	1.0	18.6	19.5	18.5	20.6	18.7	18.2			29.5	29.6		
13	♂EDB	95	116	91	111	99	121	5.4	5.8	6.7	7.1	4.1	4.6							40.0	40.0	33.8	34.2	46.1	45.5

TABLE III—*Continued.*

No.	Subject.	Sugar			Uric acid			Urea nitrogen			Non-protein nitrogen.		
		Whole blood.	Plasma	Corpuscles	Whole blood.	Plasma	Corpuscles	Whole blood	Plasma	Corpuscles	Whole blood	Plasma.	Corpuscles.
14	♀GK	86 102	81 101	94 103		3.6 4.0					32.5 32.5	27.4 27.9	40.2 38.2
17	♂FSE				4.45 4.65	6.4 6.85	2.1 1.9						
18	♂CEH				5.4 5.75	6.1 7.7	3.9 3.3						
Averages....		95 131	91 126	102 134	4.2 4.5	4.3 5.2	3.4 2.6	20.6 20.2	20.6 21.2	18.8 18.2	38.3 38.1	31.4 31.7	47.5 46.7

Subject No.	Preformed creatinine.			Total creatinine			Specific gravity	Relative viscosity		Relative corpuscle volume	Hemoglobin ratio.
	Whole blood.	Plasma.	Corpuscles.	Whole blood	Plasma	Corpuscles		Whole blood	Plasma		
										per cent	
1										42 43	
1′	1.1 1.3	1.1 1.3	1.1 1.3					4.90 5.22		44.4 44.6	
1″	1.3 1.4	1.3 1.3	1.3 1.5	5.1 5.1	2.8 2.8	8.0 7.8	1.054 1.054	4.32 4.31		43.9 45.8	100 102
2										38.5 40.2	
2′	1.2 1.2	1.1 1.15	1.3 1.25	5.9 7.0	3.2 3.6	10.0 11.6	1.055 1.060	4.18 4.39		40.0 42.3	
3	1.2 1.2							5.0 5.4		37.6 38.4	
4	1.4 1.4							5.38 5.71		45.5 50.0	
5	1.2 1.3							4.85 5.23		40.7 40.1	

TABLE III—*Concluded.*

Subject No.	Preformed creatinine.			Total creatinine			Specific gravity	Relative viscosity		Relative corpuscle volume.	Hemoglobin ratio
	Whole blood.	Plasma.	Corpuscles	Whole blood.	Plasma	Corpuscles		Whole blood.	Plasma		
										per cent	
6	1.3							4.61		40.9	
	1.5							5.11		44.4	
7	1.3							4.81		45.1	
	1.4							5.35		47.7	
8	1.3			5.4			1.058	4.79			
	1.35			6.0			1.060	4.91			
9	1.5	1.5	1.5				1.058	4.70		44.4	
	1.5	1.5	1.5				1.063	5.17		43.3	
9′	1.3	1.3	1.3	5.1	3.0	7.9	1.056	4.63	1.64	42.4	
	1.3	1.3	1.3	5.1	3.1	7.6	1.059	4.63	1.67	44.4	
10	1.2	1.15	1.25	4.8	3.2	7.5		4.05		39.2	
	1.35	1.25	1.5	5.2	3.3	8.0		4.35		40.1	
10′	1.2	1.25	1.15	5.3	2.5	9.3	1.0555	4.44	1.53	41.4	100
	1.2	1.2	1.2	5.7	2.7	9.3	1.061	5.02	1.68	45.5	107
11	1.35	1.3	1.4	5.7	2.6	8.9		4.44	1.45	49.5	100
	1.4	1.35	1.45	5.6	3.1	7.8		5.14	1.55	53.0	105

12	1.05 1.15	1.0 1.1	1.1 1.2				056 1.061	4 31 5.55		43.5 47.0	
13		1.2 1.2		5.9 6.4	3.0 3.1	8.8 9.5	.063 1.064	5.00 5.30	1.61 1 67	50 3 51.3	100 102
14				4.8 5.3	3 1 3.1	7.4 8.8	.053 1.058	3 60 4 28	1 49 1 57	39.8 44.5	100 108
17										45.5 44.6	
18									1.61 1.69	40.5 43.8	
Averages...	1.26 1.33	1.22 1 26	1.30 1 36	5 3 5.7	2 9 3.1	8 5 8.7	056 1 060	4 59 5.00	1 56 1 64	42.8 44.7	100 105

***Showing the effect of short, intense exercise** upon certain blood co stituents. In each column the upper, left-hand **number applies to the sample taken** before the exercise, while the lo er. right-hand one applies to the sample taken **afterwards.** The exercise consisted of running rapidly up a flight of sta rs until nearly exhausted. The work varied from **30,000 to 100,000** foot pounds in an average time of 10 to 15 minutes. l values are expressed in mg. per 100 cc. of blood, except in cases otherwise apparent.

TABLE IV.*

Long Period of Exercise.

No.	Subject.	Sugar.			Uric acid.			Urea nitrogen			Non-protein nitrogen.		
		Whole blood.	Plasma	Corpuscles.	Whole blood	Plasma	Corpuscles.	Whole blood	Plasma	Corpuscles	Whole blood.	Plasma.	Corpuscles.
1	♂NWR	96 68	95 46	97 95	2.7 3.2	2.8 4.3	2.6 1.9	18.2 18.2	20.4 20.0	15.0 16.0	37.8 43.3	26.4 28.6	54.4 61.1
1′	♂NWR	93 88	84 67	104 113	4.0 4.8	4.8 6.6	3.0 2.6	15.3 17.8	16.1 18.9	14.3 16.5	32.3 36.6	22.1 24.6	45.3 50.6
11	♂ABS	92 94	79 88	106 101	7.3 7.0	8.3 9.6	6.2 3.9	18.8 19.6	18.8 19.6	18.8 19.6	44.9 38.9	30.3 28.1	60.7 51.9
13	♂EDB	98 100	104 89	91 112	5.6 6.4	7.7 8.6	3.2 4.0	20.5 23.2	22.2 24.8	18.6 21.4	45.8 46.3	28.3 32.3	65.8 61.8
6	♂ELG	102 99	97 92	109 109	5.6 6.8	7.3 8.4	3.0 4.5	17.3 19.7	19.7 21.8	13.7 17.0	37.1 38.2	24.7 25.3	55.8 56.7
15	♂WAC	117 100	115 80	120 125	4.0 4.9	3.1 6.0	5.1 3.5	18.8 19.2	17.8 17.9	20.1 20.8	43.8 49.6	26.5 28.7	65.4 75.6
14	♀GK	100 114	94 90	109 149	2.2 4.1	2.7 5.1	1.4 2.7	21.4 25.3	26.6 26.4	(13.4) 23.7	36.1 46.6	28.4 34.7	47.9 63.7
3	♀BBR	88 90	91 92	84 87	3.0 3.9	3.9 4.8	1.7 2.7	21.2 23.7	21.0 24.0	21.5 24.1	35.7 40.5	29.5 33.3	44.5 50.4
16	♀IGM	105 94	104 87	107 106	2.0 2.7	2.6 3.0	1.0 2.2	21.0 21.1	21.5 21.8	20.2 19.9	33.6 35.4	29.9 31.5	39.8 42.0
Averages.........		99 94	96 81	103 111	4.0 4.9	4.8 6.3	2.9 3.1	19.2 20.9	20.5 21.7	17.4 19.9	38.6 41.7	27.3 29.7	53.1 57.1

Subject No.	Preformed Creatinine.			Total Creatinine			Specific gravity	Relative viscosity		Relative corpuscle volume.	Hemoglobin ratio.
	Whole blood.	Plasma.	Corpuscles.	Whole blood	Plasma	Corpuscles		Whole blood	Plasma		
										per cent	
1	1.3 1.4	1.3 1.35	1.3 1.45	5.2 5.4	2.8 2.8	8.7 8.5	1.058 1.062	4.71 5.50	1.49 1.54	40.8 45.3	100 104
1′	1.2 1.3	1.1 1.2	1.3 1.4	4.4 4.5	2.05 2.05	7.4 7.4	1.061 1.064	4.83 5.64		43.9 45.5	
11	1.2 1.2	1.0 1.0	1.4 1.4	4.7 4.6	2.3 2.2	7.3 7.5	1.060 1.060	4.88 4.93		48.0 45.5	
13	1.2 1.2	1.1 1.1	1.3 1.3	4.9 4.8	2.1 2.1	8.1 7.8	1.062 1.063	5.93 5.42		46.8 47.5	100 100
6	1.1 1.1	1.0 1.0	1.2 1.2	4.1 4.3	2.3 2.4	6.8 7.0	1.059 1.059	4.64 4.64		39.9 41.1	
15	1.3 1.4	1.3 1.3	1.3 1.5	4.7 5.2	2.7 3.5	7.2 7.3	1.058 1.059	4.61 5.03		44.4 44.6	100 100
14	1.4 1.5	1.3 1.3	1.55 1.8	5.5 6.0	2.8 3.2	9.6 10.0	1.055 1.058	3.95 4.28	1.47 1.48	39.5 41.0	100 103
3	1.1 1.2	1.0 1.1	1.2 1.3	5.0 5.3	2.4 2.4	8.7 9.3	1.058 1.059	5.10 5.46	1.64 1.63	41.2 42.0	100 105
16	1.1 1.1	1.1 1.1	1.1 1.1	4.1 4.2	2.3 2.4	7.1 7.3	1.051 1.055	3.58 4.13	1.58 1.62	37.3 37.0	100 107
Averages....	1.21 1.27	1.13 1.16	1.29 1.38	4.7 4.9	2.4 2.6	7.9 8.0	1.058 1.060	4.69 5.01	1.54 1.57	42.4 44.4	100 103

* Showing the effect of long, moderate exercise (2 to 3 hours of fairly rapid bicycle riding) upon certain blood constituents. As in Table III, the upper left-hand numbers in each column apply to the first sample and the lower right-hand ones to the second.

TABLE V.

Comparison of Averages.

Period.	Sugar.				Uric acid.				Urea nitrogen.			
	Whole blood.	Plasma.	Corpuscles.	Plasma/Corpuscles	Whole blood	Plasma	Corpuscles	Plasma/Corpuscles	Whole blood	Plasma.	Corpuscles.	Plasma/Corpuscles
Short.	95 131	91 126	102 134	0.9 0.9	4.2 4.5	4.3 5.2	3.4 2.6	1.3 2.0	20.6 20.2	20.6 21.2	18.8 18.2	1.1 1.2
Long.	99 94	96 81	103 111	0.9 0.7	4.0 4.9	4.8 6.3	2.9 3.1	1.7 2.0	19.2 20.9	20.5 21.7	17.4 19.9	1.2 1.1
	Non-protein nitrogen.				Preformed creatinine				Total creatinine			
	Whole blood.	Plasma.	Corpuscles	Plasma/Corpuscles	Whole blood	Plasma	Corpuscles	Plasma/Corpuscles	Whole blood	Plasma.	Corpuscles	Plasma/Corpuscles
Short.	38.3 38.1	31.4 31.7	47.5 46.7	0.7 0.7	1.26 1.33	1.22 1.26	1.30 1.36	0.9 0.9	5.3 5.7	2.9 3.1	8.5 8.7	0.35 0.35
Long.	38.6 41.7	27.3 29.7	53.1 57.1	0.5 0.5	1.21 1.27	1.13 1.16	1.29 1.38	0.9 0.85	4.7 4.9	2.4 2.6	7.9 8.0	0.3 0.3

	Cholesterol.				Specific gravity	Viscosity.		Relative corpuscle volume	Hemoglobin ratio.
	Whole blood.	Plasma.	Corpuscles.	Plasma / Corpuscles		Whole blood.	Plasma.		
Short.	164	148	180	0.8	1.056	4.59	1.56	42.8	100
	155	155	156	1.0	1.060	5.00	1.64	44.7	105
Long.	161	157	165	0.9	1.058	4.69	1.54	42.4	100
	154	152	161	0.9	1.060	5.01	1.57	44.4	103

The increase in plasma and corpuscle sugar was of the same order of magnitude, and the exercise accordingly had no effect upon the distribution of sugar between the two fractions.

It will immediately be observed that the distribution of sugar in all these experiments is different from the distribution relation now generally accepted as correct. The consistent fact that the sugar in the corpuscles proved to be greater in amount than that in the plasma was the subject of much concern. The recent work of Ege (5) and others seems to have shown conclusively that the concentration of sugar in the corpuscles is about three-fourths of that in the plasma. Wishart (6) insists that results showing as much sugar in the corpuscles as in the plasma are due to faulty technique. Falta and Richter-Quittner (7) have even presented results indicating that the corpuscles normally contain no sugar whatever, but their work does not seem to have been generally accepted as yet. Thus far, however, no distribution data appear to have been given on the basis of the Folin-Wu method, and the reason for the discrepancy in this respect between the latter and other methods is being further investigated in this laboratory. It may be pointed out that the normal, resting value for whole blood is no higher than the commonly accepted average, and there seems to be no explainable reason why the plasma values should be so low. That an incomplete removal of proteins in whole blood did not result in these cases from too low an acidity at least, has been demonstrated, since blood sugar values were no lower when a distinct excess of sulfuric acid had been added.

Whatever the true explanation of the apparent discrepancy it will not affect the comparative nature of the values given in the tables.

When we turn to the longer period we find only one case out of nine in which there was an increase of more than 0.002 per cent, but on the contrary an average *decrease* of 0.005 per cent in the nine cases observed. The decrease was much more pronounced in the plasma, amounting to 0.015 per cent on the average, as a result of which the calculated sugar in the corpuscles increased 0.008 per cent. In only one case did the corpuscle sugar drop as much as 0.005 per cent, although the decrease in the plasma was often many times this. In fact there seems to have been a distinct tendency for the corpuscles to resist a decrease in sugar content, and this is also observed in the one case of decrease in Table III.

In other words, the longer exertion changed the distribution coefficient for sugar slightly, with the result that, on the average, there was relatively more sugar in the corpuscles after the exercise than before. This conclusion must be taken advisedly, however, until the matter of normal distribution of sugar, using the Folin-Wu method, has been cleared up.

Uric Acid.

Both the short and the long exercise caused an increase in uric acid which was greater, on the average, in the plasma than in the whole blood, so that in many cases the calculated uric acid in the corpuscles actually decreased. This is the most distinct effect upon distribution which was observed in any of the materials, there being no doubt that there was relatively less uric acid in the corpuscles after exercise than before.

The further increase in uric acid after the completion of the exercise has been noted in the one case in Table III. Unfortunately it was impossible to follow this subsequent change in the rest of the subjects, but a more intensive investigation of this point will be made later.

Urea and Non-Protein Nitrogen.

The short period of exercise had no particular effect upon either urea or non-protein nitrogen, nor upon their distribution between corpuscles and plasma. Not only do the averages show close agreement but there are few wide variations in the individual results. These determinations were made in triplicate and each value generally represents the average of three results. The urea includes, as usual, the ammonia nitrogen.

The longer period of exercise apparently had a very slight augmenting effect upon the urea and a somewhat greater one upon the non-protein nitrogen. There was no change in the distribution coefficient of either urea or non-protein nitrogen, however.

Preformed and Total Creatinine.

The values for preformed creatinine remained remarkably constant in both periods of exercise. This is no more than would be expected from the repeatedly observed constancy of creatinine as a blood constituent. There seemed to be a very slight tendency to increase, however, since a number of cases showed a noticeable rise, but no instance of a fall was observed. This increase amounted to less than 0.1 mg. on the average and was equally distributed between corpuscles and plasma.

Total creatinine was slightly increased, on the average, in both periods of exercise, although the distribution coefficient remained unchanged. The increase was not invariable, however; in several cases there was no observable change but in only two was there even a slight decrease.

The colorimetric comparison in the creatinine determinations is by far the most difficult of any, giving a certain element of uncertainty to small differences. The effect of exercise upon the preformed and total creatinine of the blood must therefore be considered more or less doubtful.

Number of Red Corpuscles.

Along with the other determinations red blood counts were made on the first thirteen subjects in the short period. The time consumed was so considerable and the results themselves so uncertain and variable in unskilled hands that, after establishing the fact that an actual increase occurred, the counts were not made beyond this point. Though the individual results are not given in the table the average of 13 sets of determinations showed an increase from 5.36 million to 5.55 million per c.mm. This increase, though small, is in fair agreement with those reported by Hawk (8) and by Schneider and Havens (9) over relatively long periods of exercise.

Specific Gravity, Corpuscle Volume, and Hemoglobin.

Except in three cases in which no change was observed the specific gravity increased invariably in both periods of exercise. This increase amounted, on the average, to 0.004 and 0.002 in the two periods.

With very few exceptions the relative volume of the corpuscles increased noticeably.

The relative hemoglobin values, or "hemoglobin ratios," showed a slight increase in most of the cases observed. This increase averaged 3 to 5 per cent.

These changes in specific gravity, corpuscle volume, and hemoglobin content are undoubtedly to some extent attributable to increases in the number of corpuscles. An increase in the corpuscle volume has also been shown to result from the higher carbon dioxide content of the blood.

Viscosity.

With very few exceptions the viscosity of the whole blood increased considerably in both periods. The reason for this increase has been pointed out by Adam (10), who has shown that the blood viscosity is dependent not only upon the concentration of the blood and the number of corpuscles, but especially upon the carbon dioxide content. Since the latter fact was not fully appreciated until after this work was nearly completed no careful precautions were taken to make the viscosity measurements under similar conditions of carbon dioxide tension. In all cases the blood was mixed sufficiently to prevent settling of the corpuscles, but not shaken enough to result in the loss of much carbon dioxide. The values are, therefore, purely qualitative.

Adam has shown that the viscosity of the plasma is also affected by the carbon dioxide content and from his results it would seem that the slight increases in the plasma viscosity found in this work may be traced almost wholly to that source, since the plasma was not arterialized by shaking or passage of oxygen.

Cholesterol.

The data on cholesterol are collected separately in Table VI. The relative corpuscle volumes are not included, since most of the subjects are the same as those in Tables III and IV.

The changes in cholesterol were generally small and not thoroughly consistent. On the whole, however, there was a general tendency to decrease, more noticeable in the short period than in the long, to which the corpuscles contributed more than the plasma. Not only did the average show a slight decrease but in only three cases, in each period, was there an increase.

TABLE VI.

Cholesterol per 100 cc.

Short period.					Long period.				
Subject.		Whole blood	Plasma	Corpuscles	Subject		Whole blood.	Plasma	Corpuscles.
		mg.	*mg.*	*mg*			*mg*	*mg*	*mg.*
1	♂NWR	162 155	155 166	171 142	1	♂NWR	159 136	167 131	149 142
2	♀ACA	225 192			1′	♂NWR	163 141		
9	♂WGB	136 148	108 155	174 139	6	♂ELG	180 144	124 147	244 111
11	♂ABS	155 155			11	♂ABS	143 150	142 156	145 143
12	♂MWG	130 124			13	♂EDB	154 210	168 147	137 288
13	♂EDB	180 140	155 174	205 108	14	♀GK	150 144	162 139	132 151
14	♀GK	165 157	175 158	150 156	15	♂WAC	147 142	166 177	123 99
16	♀IGM	161 167	158 161	166 177	3	♀BBR	169 169	161 177	180 158
17	♂FSE	150 156	131 150	173 163	16	♀IGM	173 178	173 180	173 175
18	♂CEH	157 138	141 119	180 162	5	♀MEC	153 125	158 149	146 89
19	♀HCM	150 150	118 122	199 188	20	♀MJH	180 160	150 113	222 221
20	♀MJH	150 132	155 145	142 113					
21	♂RWS	212 204	187 196	238 213					
Averages....		164 155	148 155	180 156			161 154	157 152	165 161

Considerations Bearing on Blood Volume.

There are a number of considerations which substantiate the conclusion that the total blood volume underwent no considerable change during the course of these experiments: (1) The hemoglobin content did not increase much over 5 per cent in any case, which can to some extent at least be attributed to an increase in the number of red corpuscles which is known to have occurred. (2) Increases in specific gravity were slight and may also be explained as in (1). (3) The increase in the plasma viscosity, which would quickly reflect any considerable change in the blood concentration, was slight, and it is known that a considerable portion of this increase was due to a higher content of carbon dioxide. (4) The values for preformed creatinine, recognized as an especially constant blood constituent, showed very little increasing tendency. It is very likely that any considerable change in the blood volume would be here apparent.

The combined evidence from these different sources makes it practically conclusive that changes in blood volume cannot be regarded as important disturbing influences in the validity of the analytical results of these experiments.

SUMMARY.

1. An investigation was undertaken on twenty-one human subjects to determine the changes produced by severe muscular exercise upon the following constituents of blood and plasma: Non-protein nitrogen, urea, sugar, uric acid, preformed and total creatinine, cholesterol, and hemoglobin, as well as specific gravity, viscosity, and the number and relative volume of corpuscles.
2. Two types of exercise were employed, representing short, strenuous effort and longer, more tedious work.
3. Short, strenuous exercise was invariably found to increase the blood sugar concentration both in plasma and corpuscles, while a longer period of exercise was generally accompanied by a drop in blood sugar, which was greater in the plasma than in the whole blood.
4. Both kinds of exercise were accompanied by a small increase in uric acid, of about the same order, which was greater in the plasma than in the whole blood.

5. Short, strenuous exercise had no effect upon urea or non-protein nitrogen, but longer work increased both slightly, in whole blood as well as plasma.

6. In both types of exercise the total creatinine increased very little, while the preformed creatinine underwent almost no change.

7. It is shown conclusively that there were no considerable changes in the total blood volume during the muscular exercise and that variations in the concentration of the blood are not, therefore, disturbing factors in the above conclusions.

8. Cholesterol was found to decrease very slightly, although results were not thoroughly consistent. The decrease seemed to be somewhat more noticeable in the corpuscles than in the plasma.

9. The specific gravity, hemoglobin, and the number and relative volume of corpuscles were found to increase during the periods of exercise. The viscosity of the whole blood was found to increase considerably and that of the plasma slightly.

10. Some incomplete data are given suggesting that total nitrogen is increased in the blood by exercise and that urea, non-protein nitrogen, and uric acid continue to increase for some time after a work period, while the sugar concentration, on the other hand, returns to normal within 2½ hours.

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BIBLIOGRAPHY.

1. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
2. Myers, V. C., and Wardell, E. L., *J. Biol. Chem.*, 1918, xxxvi, 147.
3. Cohen, B., and Smith, A. H., *J. Biol. Chem.*, 1919, xxxix, 489.
4. Sundstroem, E. S., and Bloor, W. R., *J. Biol. Chem.*, 1920–21, xlv, 157.
5. Ege, R., *Biochem. Z.*, 1920, cxi, 189.
6. Wishart, M. B., *J. Biol. Chem.*, 1920, xliv, 563.
7. Falta, W., and Richter-Quittner, M., *Biochem. Z.*, 1919, c, 148.
8. Hawk, P. B., *Am. J. Physiol.*, 1903–04, x, 384.

9. Schneider, E. C., and Havens, L. C., *Am. J. Physiol.*, 1914–15, xxxvi, 239.
10. Adam, H., *Z. klin. Med.*, 1909, lxviii, 177.

More or less complete bibliographies of the general subject of fatigue will be found in the following:

Brösamlen, O., and Sterkel, H., *Deutsch. Arch. klin. Med.*, 1919, cxxx, 358.
Burian, R., *Z. physiol. Chem* , 1904–05, xliii, 532.
Grote, L., Ueber die Beziehungen der Muskelarbeit zum Blutzucker, Halle, 1918.
Hastings, A. B., *Bull. Hyg. Lab., U. S. P. H.*, 1919, xxxiv, 1682.
Lee, F. S., *J. Am. Med. Assn.*, 1906, xlvi, 1491; *Am. J. Physiol.*, 1907, xviii, 267; 1907–08, xx, 170.
Lichtwitz, L , *Berl. klin. Woch.*, 1914, li, 1018.
von Moraczewski, W., *Biochem. Z.*, 1915, lxxi, 268.
Reach, F., *Biochem. Z.*, 1911, xxxiii, 436.
Scaffidi, V., *Biochem. Z.*, 1911, xxx, 473.
Scott, E. L., *Bull. Hyg. Lab., U. S. P. H.*, 1918, xxxiii, 605.
Scott, E. L., and Hastings, A. B., *Bull. Hyg. Lab., U. S. P. H.*, 1920, xxxv, 2445.
Sherman, H. C., *J. Am. Chem. Soc.*, 1903, xxv, 1159.
Spaeth, R. A., *J. Ind. Hyg.*, 1919, i, 1.
Weiland, W., *Deutsch. Arch. klin. Med.*, 1908, xcii, 223.

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